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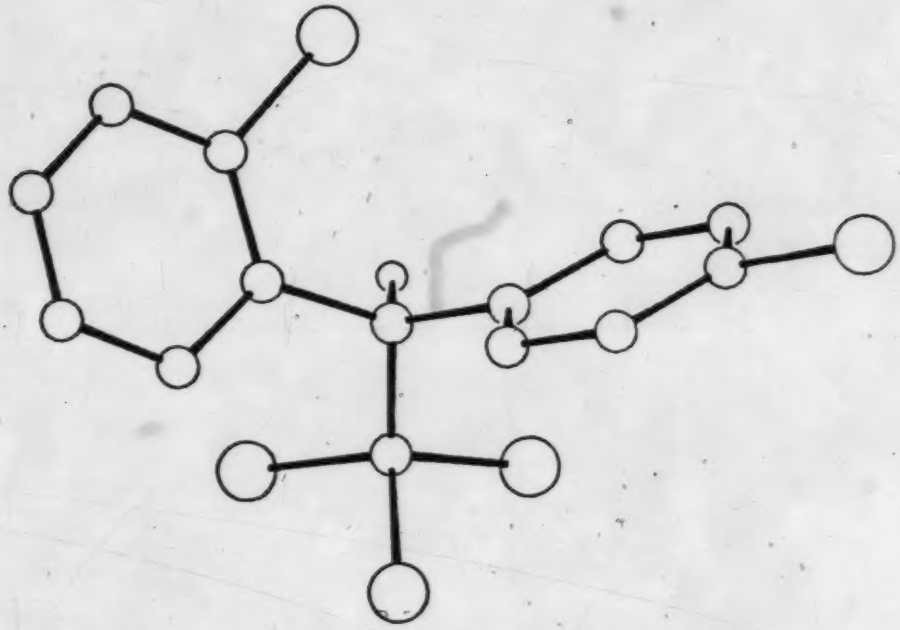

(R)-(-)-o,p'-DDT

(Drawing courtesy of Dr. M. Cowie and Dr. R. A. Smith).

THE UNIVERSITY OF ALBERTA

THE RESOLUTION AND ESTROGENIC EFFECTS OF THE ENANTIOMERS OF *o,p'*-DDT

by

© WILLIAM ALLAN MCBLAIN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "The Resolution and Estrogenic Effects of the Enantiomers of *o,p'*-DDT" submitted by William Allan McBlain in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The commercial insecticide, technical grade DDT, contains varying ratios of *p,p'*-DDT and (±)-*o,p'*-DDT as well as several other compounds. Racemic *o,p'*-DDT has been resolved into its enantiomeric forms using the classical technique of diastereomeric salt formation. Aromatic mononitration of (±)-*o,p'*-DDT was followed by reduction to the corresponding amine and formation of the (+)-10-camphorsulfonate salts. These diastereomeric salts were separated by fractional crystallization and fractional reflux techniques yielding regenerated amines with specific rotations of -170.1 and 166.4°. Deamination of the amines by treatment of their diazonium salts with hypophosphorous acid resulted in enantiomeric *o,p'*-DDT with specific rotations of -17.9 and 17.7°.

The asymmetry of *o,p'*-DDT was not reported until 1975 although it has been known since 1968 that (±)-*o,p'*-DDT has estrogenic activity in both avian and mammalian systems. In the present study the levo enantiomer of *o,p'*-DDT has been found to be more active than the dextro form in inducing increases in the estrogen-sensitive parameters, uterine wet weights and uterine glycogen levels, in immature female rats. Similarly, in immature female Japanese quail (-)-*o,p'*-DDT elicited increased oviducal wet weights and glycogen levels while the dextro form was apparently inactive. These findings provide an illustration of the operation of biological stereospecificity and demonstrate that the estrogenic effects of (±)-*o,p'*-DDT reside with the levo form.

The mechanism by which these enantiomers exhibited differing estrogenic activities is not known. It is possible that the dextro enantiomeric form could not meet the steric requirements of the estrogen receptor sites or that the diastereomeric complexes formed by the association of the two enantiomers with the receptors had chemical and physical properties sufficiently different to be reflected in differing estrogenic activities. Other environmental xenobiotics are racemic and may act through similar biological stereospecific mechanisms.

Administration of (±)-*o,p'*-DDT to immature female Japanese quail in a dietary dose of 200 ppm had no effect on oviducal wet weights, oviducal lengths or serum calcium levels. High oral doses of (-)-*o,p'*-DDT in capsules elicited estrogenic responses in the quail and an analysis of residues of *o,p'*-DDT in subcutaneous fat and uropygial glands from birds given capsules of (-)- or (+)-*o,p'*-DDT revealed that both enantiomers of *o,p'*-DDT appeared to be transported within the female quail.

Immature male Japanese quail when switched from an 8L:16D to a 14L:10D photoperiod exhibited the expected increase in testicular wet weights and concomitant testicular maturation. While (+)-17β-estradiol was able to prevent this light-stimulated response, and elicit increased serum calcium levels, the racemic and enantiomeric *o,p'*-DDT were without effect. Adverse effects of *o,p'*-DDT on male vertebrate systems have not been demonstrated to date but the environmental significance of the estrogenic activity of this compound in females is unknown. Therefore it is recommended that the estrogenic component of technical grade DDT, (±)-*o,p'*-DDT, be removed from this insecticide.

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My interest in the problem of the resolution of (±)-*o,p'*-DDT resulted from the independent recognition of the asymmetry of this compound by Dr. F. H. Wolfe in March, 1971.

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INTRODUCTION

In 1874 Zeidler first synthesized DDT (DichloroDiphenylTrichloroethane) but it was not until 1939 that Müller discovered the insecticidal properties of this chemical (Brooks, 1974). In the two decades following Müller's discovery DDT became the most widely used synthetic insecticide ever developed (O'Brien, 1967) and extensive research was devoted to elucidating the chemical and physical properties as well as the biological effects of this compound and its analogs (Müller, 1955; 1959). More recently, concern for the potential effects of environmental residues of these and other chlorinated hydrocarbon compounds has stimulated scientists from a wide range of disciplines to reinvestigate and evaluate the interaction of these chemicals with all aspects of biological systems (Brooks, 1974).

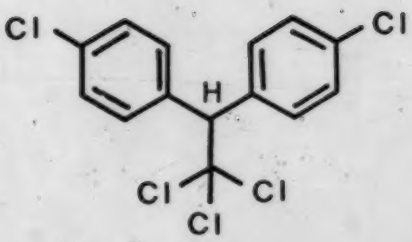
For instance, environmental contamination by chlorinated compounds has been implicated as the causitive agent in the population declines of certain birds of prey (Anderson and Hickey, 1972; Peakall, 1970; Risebrough *et al.*, 1970; Stickel, 1973). These declines are supposedly the result of an altered reproduction in these species possibly via eggshell thinning but the relationship between avian eggshell thinning and environmental pollutants has not been established definitively and the varied findings presented in the recent comprehensive reviews by Cooke (1973) and Jefferies (1973) illustrate the controversy extant in

this area of research.

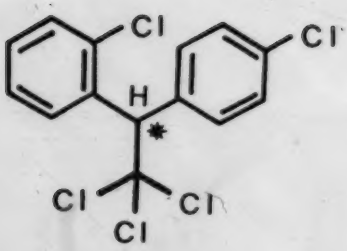
The chlorinated hydrocarbon compound most commonly associated with deleterious effects on wild avian species has been DDT. The DDT deployed in the environment is technical grade DDT, a mixture of compounds consisting primarily of about 70-80% *p,p'*-DDT (1,1'-(2,2,2-trichloroethylidene)*bis*[4-chlorobenzene]) and about 15-20% *o,p'*-DDT (1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]benzene) (Haller *et al.*, 1945) (Figure 1). It is the *p,p'*-DDT isomer and its metabolite *p,p'*-DDE (1,1'-(dichloroethenylidene)*bis*[4-chlorobenzene]) (Figure 1) that are the ubiquitous environmental contaminants thought by some investigators to have the aforementioned adverse effects on avian reproduction (Edwards, 1970; 1973). Interestingly, environmental residues of *o,p'*-DDT have been reported only rarely (Cooke, 1970) although this compound has been found to exhibit estrogenic activity in certain avian species (Kupfer, 1975).

In the period 1969-1973, amid everpresent controversy, most insecticidal uses of DDT were suspended in Canada, the United States and parts of Europe (Brooks, 1974). Following seven months of scientific testimony at the Washington Hearings in 1972, all but public health uses for DDT were terminated in the United States despite the Hearing Examiner's recommendations that several other uses be continued (Anon., 1972; Sweeney, 1972). At the present time DDT is used extensively to control malarial mosquitoes over much of the tropical world (Brooks, 1974; Jukes, 1974) and while insect resistance to DDT may eventually limit its effectiveness for this purpose, a suitable replacement has not been developed yet. In certain recent cases of insect infestations in the United States the use of DDT has been requested (Anon., 1973)

Figure 1. Structural formulae for the *p,p'*- and *o,p'*- isomers of DDT, DDE, DDD and DDA. Asterisks designate asymmetric carbon atoms.



p,p'-DDT



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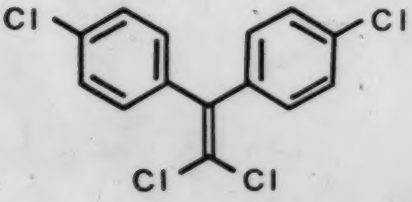

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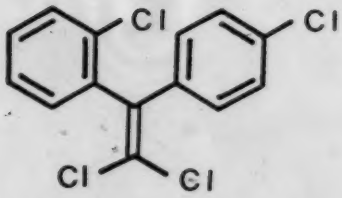

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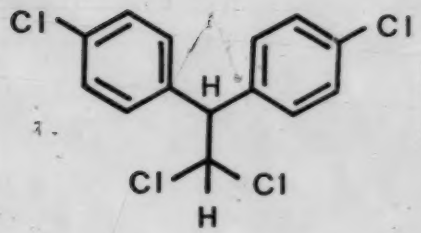

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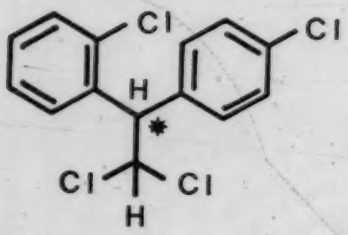

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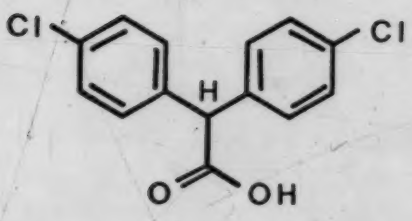
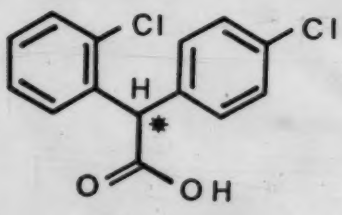


p,p'-DDA



o,p'-DDA

and in the case of the tussock moth (*Hemerocampa pseudotsugata*, McDunnough) in Oregon State, permission for use in 1974 was granted with the stipulation that some indices of ecological impact be monitored (McCluskey, 1976).

Surprisingly, in spite of 37 years of intensive research on DDT, its mode of action as an insecticide is still speculative. Similarly, the short term studies of the environmental effects of DDT carried out during the past 13 years have yielded only a limited understanding of the fate and effects of this mixture of compounds in the biosphere. In view of this general lack of knowledge of DDT and in view of its continued use as an insecticide, further research on this chemical is needed.

Haller *et al.* (1945) reported that different preparations of technical grade DDT were composed of varying ratios of the two major components, *p,p'*-DDT and *o,p'*-DDT (Figure 1). Strangely, it was not reported until 1975 (McBlain and Wolfe, 1975) that the *o,p'*-DDT isomer possesses a single asymmetric carbon atom and therefore would exist as optical isomers (Appendix 1). Similarly, the analogs of *o,p'*-DDT (and in fact metabolites (Feil *et al.*, 1973; 1975)) *o,p'*-DDA (2-chloro-α-(4-chlorophenyl)benzeneacetic acid) and *o,p'*-DDD (1-chloro-2-[2,2-dichloro-1-(4-chlorophenyl)ethyl]benzene) would be racemic modifications as well (Figure 1). Therefore more appropriate notations for these compounds would be (±)-*o,p'*-DDA, (±)-*o,p'*-DDD and (±)-*o,p'*-DDT.

Optical activity has been suggested previously as a property of *o,p'*-DDT as it was theorized that restricted rotation of the aromatic rings might lead to the existence of enantiomeric forms (atropisomerism) (McKinney *et al.*, 1974). Atropisomerism is stereoisomerism caused by a

restricted rotation about a single bond and is found, for instance, in certain biphenyl compounds with bulky substituents (Eliel, 1962; Kaiser, 1974). Often a low energy of activation is required for the interconversion of such isomers and the interconversion may be so rapid as to prevent their optical resolution (Eliel, 1962). The dissymmetry of the *o,p'*-DDT molecule, however, results from the presence of the asymmetric carbon atom rather than from a restricted rotation.

Recently the optical resolution of the enantiomeric forms of certain asymmetric analogs of *p,p'*-DDT was reported (Sagar *et al.*, 1972). The four resolved analogs consisted of combinations of H or Br in one para position and Cl or F in the other para position. The reported absolute specific rotations ranged from 1.0° to 22.8°.

Many important biochemicals are optically active and exist in the natural state in one enantiomeric form only. Therefore "the majority of molecular biological processes involve interactions between asymmetric molecules" (Barker, 1971). This predominance of one enantiomer and/or the ability to utilize only one enantiomer is the result of enzyme specificity within the appropriate anabolic or catabolic processes. The concept of the enzyme active site being three dimensional and therefore having specific steric requirements provides an adequate explanation for the production or utilization of the enantiomer possessing the better molecular structure for accommodation at the enzyme active site (Barker, 1971). It may be more appropriate, however, to view the association of the inherently dissymmetric enzyme active sites with the enantiomeric substrates as the formation of diastereomeric complexes which are known to have "different chemical properties, binding constants, activation parameters and reaction rates"

(Alworth, 1972). Hence biological systems usually differentiate between the enantiomers of optically active organic compounds (Barker, 1971) and the phenomenon is called biological stereospecificity (Alworth, 1972).

It would seem that certain drug and hormone receptors also have steric requirements for specific molecules, as indicated, for example, by the differing activities of various estrogenic agents with seemingly minor structural differences (Bitman and Cecil, 1970; Solmssen, 1945). The fact that the estrogen receptor is a protein (O'Malley and Means, 1974) and therefore inherently dissymmetric would imply that a stereospecific configuration of the estrogen molecule could be necessary for it to exhibit estrogenic activity. Support for this concept lies in the finding that the enantiomers of 17β-estradiol benzoate differ in their estrogenic activities (Meyerson, 1971).

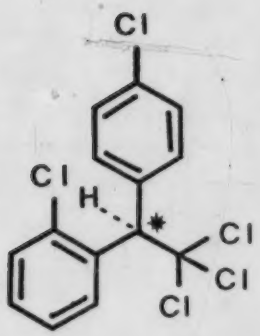
When testing the physiological responses of an animal to racemic compounds it would be desirable also to test each enantiomer independently in order to understand any observed response. The two enantiomers might act in an identical (additive), synergistic or antagonistic fashion. To evaluate the reported physiological effects of *o,p'*-DDT it seemed imperative to resolve its enantiomers and to test them individually as well as in the racemic modification.

Racemic *o,p'*-DDT has been shown to have estrogenic activity in both avian and mammalian systems. This subject has been reviewed recently by Kupfer (1975) and Ware (1975) and the following literature review describes the development of the current knowledge of this physiological effect of (±)-*o,p'*-DDT. Appendix 2 contains a tabular summary of the experiments discussed in this literature review.

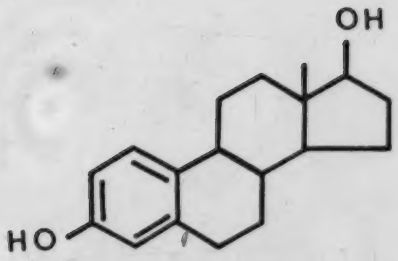
Burlington and Lindeman (1950) were the first to report a hormonal effect of DDT in an avian system. They found that *p,p'*-DDT administered to white leghorn cockerels resulted in inhibited comb and wattle development caused by retarded testes growth and suggested that the DDT had exhibited an estrogen-like action. The general health of the treated birds appeared unaltered suggesting a direct action and these authors recognized the possibility that a metabolite of the DDT could have been involved. In a later study of similar cockerels it was noted that *p,p'*-DDT did not alter plasma calcium levels as would have been expected if a strictly estrogenic effect had been observed (Burlington and Lindeman, 1952). Also in the earlier study the similarity of structure between the DDT molecule and diethylstilbestrol (Figure 2) was noted but Fisher *et al.* (1952) demonstrated that the absence of hydroxyl groups on the *p,p'*-DDT molecule apparently rendered it inactive as an estrogen. Fisher *et al.* (1952) tested estrogenic activity by the ability of the chemical to induce estrus in ovariectomized rats. Both *p,p'*-DDT and methoxychlor (2,2-*bis*(*p*-methoxyphenyl)-1,1,1 -trichloroethane) were inactive as estrogens but DHDT (2,2-*bis*(*p*-hydroxyphenyl)-1,1,1,-trichloroethane) (Figure 2) was active. The trichloromethyl group of the DHDT molecule rendered it seven times more active than the 2,2-*bis*(*p*-hydroxyphenyl)ethane analog (Figure 2) possibly by restricting ring rotation or by activating the hydroxyl hydrogens through inductive effects.

Albert (1962) demonstrated that DDT could decrease sperm production in white leghorn cockerels but he did not indicate what isomer or mixture of isomers of DDT he used. Furthermore, massive doses (3000 or 7000 ppm in the feed) were given to a small sample size (total of 7

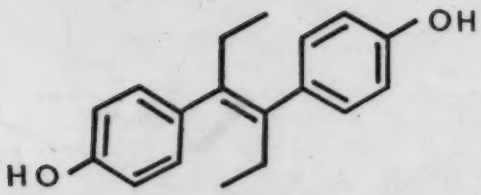
Figure 2. Structural formulae for several estrogenic compounds discussed in the text. Asterisk designates asymmetric carbon atom.



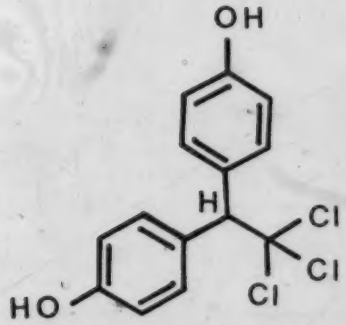
(R)-(−)-o,p'-DDT



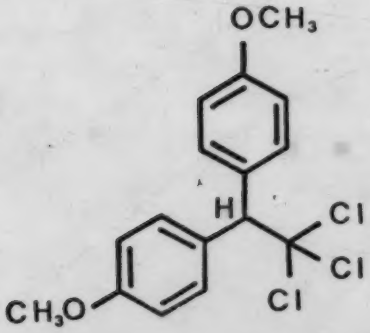
(+)-17ß-ESTRADIOL



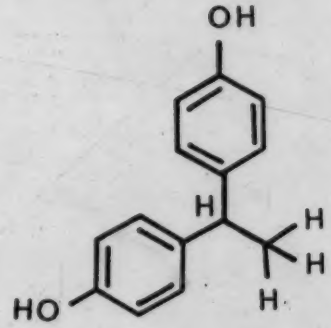
DIETHYLSTILBESTROL



DHDT



METHOXYCHLOR



DHDT ANALOG

birds) and toxicity symptoms developed shortly after the drop in sperm production was noted. This study may have indicated only that the dying birds were impaired physiologically.

True estrogenic activity of DDT was demonstrated conclusively by Levin *et al.* (1968). They found that technical grade DDT and *o,p'*-DDT increased the uterine wet weights in immature and ovariectomized female rats and that both compounds inhibited the uterine uptake of estradiol-6,7-H^3 and stimulated glucose-U-C^{14} uptake into uterine lipid, protein and RNA. Technical grade DDT was less active than *o,p'*-DDT; methoxychlor had moderate activity; *p,p'*-DDT, *o,p'*-DDD and *m,p'*-DDD were mildly active; and *p,p'*-DDD and *p,p'*-DDE had little or no activity. Bitman *et al.* (1968) confirmed these findings for *o,p'*-DDT and *p,p'*-DDT in rats and demonstrated that the uterine glycogen response was a sensitive indicator of estrogenic activity. These authors also found increased oviducal weights and glycogen levels in both chickens and Japanese quail treated with *o,p'*-DDT. The DDT treatments were compared to untreated controls as well as 17β-estradiol-treated animals and *o,p'*-DDT appeared to be about 1/1000 as estrogenically active as the estradiol in the rats and about 1/100 as active in the chickens and quail.

Cooke (1970) also gave Japanese quail *o,p'*-DDT but found neither significant changes in the weights of testes, ovaries and oviducts nor significant changes in serum calcium and lipid levels.

In this latter study the *o,p'*-DDT was administered by the im (intramuscular) route while in the former study (Bitman *et al.*, 1968) the ip (intraperitoneal) route was utilized. Since all other experimental conditions were comparable it is obvious that the route of administration for *o,p'*-DDT was an important factor in these studies.

Bitman *et al.*, (1969) reported that 100 ppm *o,p'*-DDT in the diet of Japanese quail caused a delay in the production of eggs but had no effect on the number of eggs produced. The eggs from the treated birds were lighter and had thinner eggshells containing less calcium than the controls. The birds were not given sufficient calcium in the diet, however, and when the dietary calcium of a parallel *p,p'*-DDT study was returned to the known minimal requirement, the measured eggshell parameters did not differ from the controls (Cecil *et al.*, 1971b). The *o,p'*-DDT experiment was not repeated under the conditions of adequate dietary calcium.

Lillie *et al.* (1972) found that 50 ppm of *o,p'*-DDT in the diet of chickens for 28 weeks reduced hatchability and 150 or 300 ppm for 12 weeks following 20 weeks at 5 and 25 ppm respectively, reduced the egg production. These latter treatments did not alter mortality, fertility, hatchability or progeny growth. In the same experiment the egg weights and eggshell characteristics of thickness and calcium content were unaffected (Cecil *et al.*, 1972). Thus, *o,p'*-DDT, in the laboratory studies conducted, was ineffective in producing reproductive abnormalities in these birds except in cases of relatively high doses. These findings are similar to those for technical grade DDT and *p,p'*-DDT although variations in results exist both among and within species (Cecil *et al.*, 1973; Cooke, 1973; Lillie *et al.*, 1973; Scott *et al.*, 1975).

Welch *et al.* (1969) presented the data of Levin *et al.* (1968) in more detail and observed that pretreatment of rats with carbon tetrachloride decreased the uterotropic action of *o,p'*-DDT suggesting that hepatic conversion to an active metabolite (perhaps a hydroxylated

derivative) was necessary for estrogenic activity. The DDT and its analogs were administered, however, by ip injections in DMSO (dimethylsulfoxide). Work with *p,p'*-DDT in mice in this laboratory (Lewin *et al.*, 1972) has revealed that DMSO may disperse rapidly to leave *p,p'*-DDT in the coelom; therefore the active doses reported by Welch *et al.* (1969) should not be considered definitive. Additionally, Welch *et al.* (1969) had reported that technical grade DDT administered to rats at 50 mg/kg ip in DMSO was mainly effective as an estrogenic agent because of its *o,p'*-DDT content and that its maximal uterotropic effect occurred at 6 hours. Singhal *et al.* (1970), however, found that im injections of 100 mg/kg *o,p'*-DDT in corn oil gave a maximal uterotropic effect in rats in 16 hours. This latter time factor was similar to the 18 hr response reported by Cecil *et al.* (1971a) for sc (subcutaneous) injections in olive oil but both the solvents and routes of administration undoubtedly affected the apparent times of response to the *o,p'*-DDT. Singhal *et al.* (1970) also reported that *o,p'*-DDT-induced increases in uterine carbohydrate enzymes (phosphofructokinase, aldolase, hexokinase and pyruvatekinase) were similar to those found for an active estrogen and were related to RNA and protein synthesis. It is noteworthy that *o,p'*-DDT and 17β-estradiol acted in an additive fashion while *o,p'*-DDT and progesterone were antagonistic in uterotropic and enzyme inductive activities.

Oral administration of 50 µg of *o,p'*-DDT to rats daily for 15 days caused an early vaginal opening similar to that found for 17β-estradiol but the estrogen-sensitive uterine parameters of wet weight, water, glucose and glycogen levels were unaltered (Wrenn *et al.*, 1970). In the same study it was found that up to 2.5 ppm *o,p'*-DDT in the feed

of rats from parturition to 168 days of age caused no changes in the above-mentioned parameters. In both cases, however, less DDT was administered than in the aforementioned investigations and a later study was designed using larger doses (Wrenn *et al.*, 1971b). Dietary *o,p'*-DDT at levels of 1, 10, 20, or 40 ppm, however, still did not alter the age at vaginal opening. Also after three weeks on the 10, 20 and 40 ppm *o,p'*-DDT-spiked diets the percentage of water in the uterus was lowered but such parameters as body weight, ovarian weight, uterine weight, uterine glycogen and uterine glucose were unchanged and all of these parameters were unchanged at 10, 14, 18 and 23 weeks of treatment at all the levels of *o,p'*-DDT tested. It would seem that the rats may have compensated for the DDT treatment by an increase of metabolism and/or excretion through hepatic microsomal enzyme induction. Similarly, a dietary dose of 10 ppm of *o,p'*-DDT did not affect the relative amount of endometrium, glycogen, glucose or water levels in the uteri of treated ewes (Wrenn *et al.*, 1971a).

Bitman and Cecil (1970) tested 53 compounds for estrogenic activity as measured by an 18 hour uterine glycogen response in rats. Activity was measured as the minimum dose effective in changing the glycogen levels significantly from those of the controls. Diethylstilbestrol was effective at 0.1 µg whereas *o,p'*-DDT, the most active DDT-type compound, was effective at 0.25 mg, a 2500-fold difference in activity. It was suggested by structure-activity correlations that a hydroxylated metabolite of *o,p'*-DDT was the estrogenically active chemical although this metabolite was neither recovered from the rats nor synthesized and tested for activity. The sample sizes and statistical tests used were not revealed.

Cecil *et al.*(1971a), apparently using some of the data of Bitman and Cecil (1970) reported the minimal effective estrogenic dose of *o,p'*-DDT in rats to be 0.4 mg, about 10,000 times that of diethylstilbestrol or 17β-estradiol. The maximal uterine glycogen response to *o,p'*-DDT was achieved after about 18 hours and like 17β-estradiol the effect could be blocked by a known estrogen antagonist (MER-25) suggesting similar uterine receptor sites for the chemicals. Administration of the *o,p'*-DDT over 3 and 7 day periods by the oral (up to 1000 ppm in the feed) and sc routes did not result in the same maximal glycogen response observed at 18 hours for single sc doses of *o,p'*-DDT. The 17β-estradiol elicited maximal glycogen responses for both the single and multiple doses. The authors suggested that for the 3 and 7 day studies the liver enzyme systems may have been metabolizing the *o,p'*-DDT rapidly enough to leave an insufficient amount to elicit maximal estrogenic responses. Doses of 250 or 500 ppm *o,p'*-DDT in the feed did appear to affect the uterine wet weights and glycogen content although a statistical analysis of the data was not presented.

The uterotropic activity and advancement of vaginal opening by 7 day oral doses of *o,p'*-DDT in rats was confirmed by Clement and Okey (1972). Doses of 500 ppm or more of *o,p'*-DDT in the feed caused advanced vaginal opening while 1000 ppm or more affected the uterine parameters of wet weight, dry weight and glycogen content.

Gellert *et al.*, (1972) reported that *o,p'*-DDT and *p,p'*-DDA were active estrogens while *o,p'*-DDD and *o,p'*-DDE were inactive. The compounds used in this study were administered ip in DMSO and the possible effects of this vehicle on the release of test compounds have been mentioned above. Serum LH (luteinizing hormone) levels were

reduced by *o,p'*-DDT treatment of ovariectomized rats but serum FSH (follicle stimulating hormone) levels were unaffected.

In another study using rats the uterotropic activity of *o,p'*-DDT was confirmed and the uterine wet weight increase at 6 hours was attributed to endometrial edema. By 72 hours endometrial and myometrial hypertrophy and hyperplasia and to a lesser extent, interstitial edema, were evident (Duby *et al.*, 1971). The *o,p'*-DDT did not maintain uterine weights 14 days after ovariectomy in rats given a 175 day treatment of 3 ppm of the chemical in the feed perhaps because of the low dose and hepatic hydroxylase induction. The *o,p'*-DDT did have a uterotropic effect in both normal and ovariectomized mink.

Van Tienhoven and Duby (1972) suggested that in the previous reports of the estrogenic activity of *o,p'*-DDT the results could have been caused by altered endogenous steroid metabolism through hepatic hydroxylase induction except in those studies using ovariectomized animals (Cecil *et al.*, 1971a ; Gellert *et al.*, 1972; Singhal *et al.*, 1970; Welch *et al.*, 1969). Van Tienhoven and Duby (1972) prevented the implantation of blastocysts in rats by a treatment of medroxyprogesterone-acetate and tested the possibility that *o,p'*-DDT could induce implantations as was known for 17β-estradiol. The result for *o,p'*-DDT was negative. Moreover, *o,p'*-DDT did not affect the incidence of implantations in pregnant rats. These authors concluded that *o,p'*-DDT did not possess all the attributes of a potent estrogen at doses up to 20,000 times that of 17β-estradiol.

Subcutaneous injections of 1 mg *o,p'*-DDT on days 2, 3, and 4 after birth in newborn female rats resulted in a decreased time to vaginal opening and first estrus (Heinrichs *et al.*, 1971). Following several

normal estrus cycles a persistent estrus syndrome developed in all treated rats. This syndrome is observed when either estrogens or androgens are administered to neonates and the authors suggested that some aspect of hypothalamic LH had been altered by the *o,p'*-DDT treatment. Treated rats also exhibited a lesser uterotropic response to subsequent doses of estradiol, probably because the number of available uterine estradiol receptors was decreased in some way. This decrease may have been an effect of an altered hormonal balance because of the syndrome, rather than a direct blockade by *o,p'*-DDT, since no *o,p'*-DDT or its metabolites were found in ovaries, brains or fat samples of treated animals. Later work by Gellert *et al.* (1974) demonstrated that an ip dose of *o,p'*-DDT as low as 0.1 mg on days 2, 3, and 4 after parturition could induce early vaginal opening and persistent vaginal estrus in injected females but this group as well as Campbell and Mason (1975) reported that neonatal injections of up to 3 mg had no effect in male rats with respect to testicular weight or histology. A sc dose of 2.82 µmole (1.00 mg) of *o,p'*-DDT on days 2, 3, and 4 after birth of male rats was found to be ineffective in altering the neonatal imprinting of hepatic steroid metabolism (Gustafsson and Stenberg, 1976).

Oral treatment of pregnant female rats with *o,p'*-DDT in an attempt to provide a natural exposure of neonatal animals to the chemical induced ovulatory abnormalities only in cases of very high doses of the compound (Clement and Okey, 1974). In this study 1000 ppm of *o,p'*-DDT in the feed of the dams was effective in altering the reproductive performance of female progeny while 20 and 200 ppm treatments were ineffective. Similarly *o,p'*-DDT given by gavage at 10 mg/day for days

15-19 of pregnancy was without effect on female progeny (Gellert and Heinrichs, 1975). It would seem unlikely that environmental exposure to *o,p'*-DDT could approach the levels necessary to affect neonatal hypothalamic development (Clement and Okey, 1974). Technical grade DDT at 75 or 150 ppm in the diet of mature rats did not affect the length of estrus cycles and although the 150 ppm diet was reported to alter the production of young (Jonsson *et al.*, 1976) the sample size (3) was too small for the result to be considered significant.

Doses of 50 or 100 mg/kg *p,p'*-DDT ip did decrease the percentage of implanted ova in mice possibly by altering steroidal metabolism (anti-estrogenic effect (below)) (Lundburg and Kihlström, 1973). In a similar study the percentage of implanted ova was decreased in mated mice only when both the male and female had been exposed neonatally to technical grade DDT by suckling DDT-treated dams but not when only one of the mated animals had been exposed (Kihlström *et al.*, 1975).

The ability of *o,p'*-DDT to in some ways mimic estrogens is termed an estrogenic effect while the ability of *p,p'*-DDT or *o,p'*-DDT to activate liver microsomal enzymes and thereby cause excessive endogenous steroid metabolism may be called an anti-estrogenic effect (Clement and Okey, 1972). These authors found that 100 ppm *p,p'*-DDT in the feed of rats delayed estradiol-stimulated vaginal opening and as little as 50 ppm *o,p'*-DDT decreased estradiol-stimulated uterine glycogen levels. Örberg *et al.* (1972) found that *p,p'*-DDT could lengthen the estrus cycles in mice. These effects are anti-estrogenic in females and may occur only because the hepatic microsomal enzyme and endogenous or exogenous steroid levels have not equilibrated (Fahim *et al.*, 1970). Similarly, induction of these enzymes may

have indirect estrogenic effects in male animals by altering androgen metabolism. The *o,p'*-DDT is not only estrogenic but through its ability to activate hepatic microsomal enzymes (below) it may also act in an anti-estrogenic fashion.

The activity of hepatic microsomal enzymes may be elevated by the administration of several chemicals including DDT and its analogs. This induction occurs both in mammals (Street, 1969; Conney *et al.*, 1973) and birds (Abou-Donia and Menzel, 1968c; Nowicki and Norman, 1972; Peakall, 1967) and involves a *de novo* synthesis of mixed function oxidase (hydroxylase) enzymes.

Most of the early work involving hepatic microsomal enzyme induction by DDT has involved either technical grade DDT or *p,p'*-DDT but recent work has included *o,p'*-DDT. Bitman *et al.* (1971b) measured the effectiveness of *o,p'*-DDT and *p,p'*-DDT as microsomal stimulators in rats and quail by observing changes in pentobarbital-induced sleeping times. In rats *o,p'*-DDT was less effective than *p,p'*-DDT in reducing sleeping times while the *o,p'*- and *p,p'*- isomers of DDE were more effective than either DDT isomer and the *o,p'*- and *p,p'*-isomers of DDD were the least effective. Relative to the *p,p'*- isomers all of the *o,p'*- isomers were present in low quantities in body lipid indicating a rapid metabolism and/or excretion. In contrast, the pentobarbital-induced sleeping times of Japanese quail were increased by both *o,p'*- and *p,p'*-DDT administration probably because of the competitive metabolism of DDT and pentobarbital and once again the relatively rapid metabolism of the *o,p'*-DDT was reported.

Abernathy *et al.* (1971) investigated the induction of hepatic microsomal enzymes in mice by several DDT-related compounds using

NADPH oxidation, cytochrome P-450 level and aniline hydroxylation as indices of mixed function oxidase activity. The *o,p'*-DDD did not affect the measured microsomal indices but *o,p'*-DDT significantly altered them although it was less potent than *p,p'*-DDT. *In vivo* treatment of chickens with *o,p'*-DDT increased the *in vitro* microsomal metabolism of testosterone, 4-androstene-3, 17-dione and 17β-estradiol (Nowicki and Norman, 1972). Dogs treated with *o,p'*-DDT rapidly converted it to *o,p'*-DDD and one of the two chemicals induced an increase in liver microsomal activity (Copeland and Cranmer, 1974).

The activated liver microsomal enzymes metabolize DDT and its analogs as well as the endogenous steroids. The metabolism of *p,p'*-DDT has been investigated in both avian (Abou-Donia and Menzel, 1968a; Bailey *et al.*, 1969; Ecobichon and Saschenbrecker, 1968; Menzie, 1969; 1974) and mammalian (Fries *et al.*, 1969a; Menzie, 1969; 1974; O'Brien, 1967) systems. In general, *p,p'*-DDT is converted either to *p,p'*-DDD or *p,p'*-DDE (Figure 1) by separate pathways. Most of the *p,p'*-DDD is excreted directly or metabolized through several intermediates to water soluble *p,p'*-DDA (Figure 1). The *p,p'*-DDE is not metabolized as readily although hydroxy metabolites of *p,p'*-DDE have been identified recently (Jansson *et al.*, 1975; Sundström *et al.*, 1975). Unmetabolized *p,p'*-DDT may be excreted or stored in fatty tissues.

The metabolism of *o,p'*-DDT in birds and mammals has not been investigated as extensively as that of *p,p'*-DDT and has generated some controversy. In 1964, Klein *et al.* reported a conversion of *o,p'*-DDT to *p,p'*-DDD and *p,p'*-DDT in rats and later supposedly confirmed their findings (Klein *et al.*, 1965). Ecobichon and Saschenbrecher (1968) found a similar conversion in chickens as did French and Jefferies (1969)

in pigeons. Bitman *et al.*, (1969), however, found no conversion of *o,p'*-DDT to *p,p'*-DDT in Japanese quail but reported that a 0.4% contamination of their *o,p'*-DDT by *p,p'*-DDT would have accounted for *p,p'*-DDT in their *o,p'*-DDT-treated birds. Lamont *et al.* (1970) working with mallard ducks and Wrenn *et al.* (1971a) using sheep also reported no conversion to *p,p'*-DDT in their *o,p'*-DDT-treated animals; this latter research group therefore designed a study to ascertain whether *o,p'*-DDT was isomerized to *p,p'*-DDT in selected biological systems.

It was found subsequently that *p,p'*-DDT was an impurity in commercial *o,p'*-DDT samples at levels ranging from 0.4-1.3% (Bitman *et al.*, 1971a;b). This contamination was adequate to account for the levels of *p,p'*-DDT found in rats, sheep, chickens and quail fed *o,p'*-DDT. Therefore no conversion to *p,p'*-DDT in these species was needed to account for the *p,p'*-DDT found. Pure *o,p'*-DDT isolated by column chromatography gave *p,p'*-DDT levels in rats comparable only to those in controls and radioactive *o,p'*-DDT did not give rise to radioactive *p,p'*-DDT (Bitman *et al.*, 1971a; Cranmer, 1972). Therefore it was concluded that contamination of *o,p'*-DDT by *p,p'*-DDT had led the earlier workers to conclude incorrectly that a conversion of *o,p'*-DDT to *p,p'*-DDT had taken place. The *o,p'*-DDT was metabolized and excreted much more rapidly than the *p,p'*-DDT and therefore the *p,p'*-DDT and its metabolites had accumulated to a relatively greater extent (Bitman *et al.*, 1971a).

Japanese quail also were found to metabolize *o,p'*-DDT faster than *p,p'*-DDT (Bitman *et al.*, 1969) as did mallard ducks (Lamont *et al.*, 1970) and man (Morgan and Roan, 1972). This relatively rapid

metabolism was thought to explain the apparent absence of *o,p'*-DDT and its metabolites from wildlife tissue samples and from animal tissues in laboratory studies employing technical grade DDT (Cooke, 1970).

The relatively low acute toxicity of *o,p'*-DDT compared to that for *p,p'*-DDT found in both birds and mammals (Browning *et al.*, 1948; Ecobichon and Saschenbrecker, 1968; Gill *et al.*, 1970; Okey and Page, 1974) may reflect its relatively more rapid metabolism and excretion by these animals (Bitman *et al.*, 1971a). This latter group emphasized the technical problems involved in the separation and identification of mixtures of the various *o,p'*- and *p,p'*- derivatives of the parent DDT compounds. Only the recently developed analytical techniques have allowed for a more complete description of the metabolism of *o,p'*-DDT (Feil *et al.*, 1973; 1975).

Klein *et al.* (1964) fed rats *o,p'*-DDT and analysed the liver for residues but reported *o,p'*-DDT as the only *o,p'*- compound present. Mendel *et al.* (1967) reported the conversion of *o,p'*-DDT to *o,p'*-DDD by *Aerobacter aerogenes* but the total of these two compounds recovered was less than 80% of the original *o,p'*-DDT suggesting the possibility of other unidentified metabolites. Rumen microorganisms similarly converted *o,p'*-DDT to *o,p'*-DDD (Fries *et al.*, 1969b).

Apparently when fed to pigeons, *o,p'*-DDT was metabolized and excreted since its levels in liver, muscle and fat tissues all declined with time but no metabolites were found (French and Jeffries, 1969). A *post mortem* conversion to *o,p'*-DDD was found, however, in the livers and muscle of these birds. Rats fed *o,p'*-DDT have both *o,p'*-DDT and *o,p'*-DDD present in body fat (Bitman *et al.*, 1971b; Cranmer, 1972) and

pigeon liver preparations also can convert *o,p'*-DDT to *o,p'*-DDD (Hassall and Manning, 1972).

The first detailed analysis of the metabolism of a single oral dose of *o,p'*-DDT in the rat resulted not only in the identification of *o,p'*-DDD as a metabolite of *o,p'*-DDT but also *o,p'*-DDA and several hydroxylated and methoxylated derivatives of each of these compounds (Feil *et al.*, 1973). A later study using chickens revealed *o,p'*-DDE as a further metabolite of *o,p'*-DDT (Feil *et al.*, 1975) but in both this and the 1973 study metabolism of the *o,p'*-DDT by gut microflora was probable since the metabolites were isolated from urine and feces. The estimation that each metabolite represented only 1-3% of the original dose would suggest that if one of the metabolites was the active estrogen its potency might be more comparable to that of 17β-estradiol. Similar hydroxylations of PCB's (polychlorinated biphenyls) (Hutzinger *et al.*, 1972; Jensen and Sundström, 1974), *p,p'*-DDE (Sundström *et al.*, 1975) and dieldrin (Krampitz and Hardebeck, 1973) in the rat have been observed as well.

Zeitoun and Makar (1973 a;b) reported that pretreatment with SKF 525-A, a known microsomal enzyme inhibitor, increased the uterotropic activity of *o,p'*-DDT in rats while phenobarbital, a microsomal enzyme inducer had no effect on this parameter. Therefore, they suggested that *o,p'*-DDT did not act via an active metabolite in contrast to previous suggestions (Bitman and Cecil, 1970; Welch *et al.*, 1969) that a hydroxylated metabolite was involved. Zeitoun and Makar (1973b), however, have ignored their own statistical findings in reporting a reduced uterotropic activity and as well they suggest naively that a conversion of *o,p'*-DDT to *p,p'*-DDT may have been involved in their

study.

Binding to rat uterine estrogen receptors has been investigated for *o,p'*-DDT and a number of its analogs (Forster *et al.*, 1975; Nelson, 1974). The *in vitro* inhibition of ^{3}H-estradiol binding by these compounds was correlated to their *in vivo* ability to increase uterine wet weights suggesting that *o,p'*-DDT itself and not a metabolite may have been the active estrogen.

Technical grade DDT was found to inhibit the uptake of testosterone by the prostate glands of mice possibly via microsomal enzyme induction although receptor site competition may have been involved; similar amounts of the DDT were not directly estrogenically active in female mice (Smith *et al.*, 1972; Lloyd *et al.*, 1974). Wakeling and Visek (1973) reported that *o,p'*-DDT inhibited the *in vitro* binding of 5α-dihydrotestosterone to specific receptor proteins in the rat prostate gland, supporting the receptor site competition theory.

In spite of its widespread dispersal as a component of technical grade DDT, the apparent absence of *o,p'*-DDT from environmental samples (Cooke, 1970) would seem to indicate that its estrogenicity would not have a long term effect on wildlife populations. This may be an oversimplification of the situation. The inability to isolate *o,p'*-DDT and its metabolites from environmental samples has been attributed to its rapid metabolism (Bitman *et al.*, 1969; Fries *et al.*, 1969a), however, the possibility that undetected hydroxylated metabolites of *o,p'*-DDT (Feil *et al.*, 1973; 1975), with unknown physiological effects, could be present in wildlife exposed to technical grade DDT cannot be excluded at the present time. Hydroxy metabolites probably would be lost using the routine procedures for sample cleanup during pesticide residue

analysis and would be undetected by conventional gas liquid chromatographic techniques. Unfortunately, neither the effects nor the ultimate fate of *o,p'*-DDT and/or its metabolites in the environment have been delineated.

In an attempt to gain further knowledge of the chemistry and estrogenicity of *o,p'*-DDT the present study was designed with two major objectives in mind : the resolution of the optical isomers of *o,p'*-DDT was to be carried out; and the resulting enantiomers were to be tested for their relative estrogenic activities in mammalian and avian systems.

Since *p,p'*-DDA has some estrogenic activity (Gellert *et al.*, 1972) it was of interest to learn whether *o,p'*-DDA was an active estrogen and if its enantiomers differed in activity. While *o,p'*-DDA was prepared early in the investigations of the chemistry of DDT and its analogs (Cristol and Haller, 1945) its asymmetry has been described only recently (McBlain and Wolfe, 1975).

Racemic *o,p'*-DDD is used in the treatment of Cushing's syndrome and adrenocortical carcinoma (Appendix 3; Hoffman and Mattox, 1972; Lubitz *et al.*, 1973; Sizonenko *et al.*, 1974; Straw and Hart, 1975). The main deterrents to its use are the toxic side effects produced by the high doses required to obtain clinical responses. This problem might be overcome if one enantiomer is responsible for the observed beneficial effects of the drug and alone is not responsible for the toxic side effects. Therefore the resolution of *o,p'*-DDD could have an immediate and important practical application. While the resolution of *o,p'*-DDD was not attempted as a part of this research project it was hoped that a method useful for the resolution of *o,p'*-DDT might

be applicable to medically important *o,p'*-DDD.

Two reviews dealing with the resolution of racemic compounds (Boyle, 1971; Wilen, 1971) as well as a list of compounds previously resolved (Wilen, 1972) were consulted to find an appropriate route for the resolution of (±)-*o,p'*-DDT.

Louis Pasteur first resolved tartaric acid by discovering that the enantiomers of the sodium ammonium salts crystallized differently from "dilute solutions" (Alworth, 1972). The crystallization method of optical resolution is still useful but simple crystallization of *o,p'*-DDT would not have resolved the enantiomers since the crystal structure revealed in a report by DeLacy and Kennard (1972) indicated that the *o,p'*-DDT crystals had a centre of symmetry and therefore the two enantiomers would not have crystallized differentially (Cowie, Dr. M.; personal commun.).

A biological (enzymatic) optical activation[1] or resolution was not attempted because known systems of enzymatic reactions or preferential biological degradations indicating at least partial resolutions for *o,p'*-DDT have not been described. Such systems may be developed with the recognition of optical activity in this compound. For example, the high rate of metabolism of *o,p'*-DDT relative to *p,p'*-DDT (Bitman *et al.*, 1969; Fries *et al.*, 1969a) to various derivatives (Feil *et al.*, 1973; 1975) may allow a facile isolation of optically

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[1] "Optical activation is the most general term describing methods for securing one enantiomer of a compound in excess of the other. Optical resolution refers to optical activations which originate with racemic compounds." Enzymatic optical activation refers to "biochemical asymmetric synthesis and asymmetric destruction. The latter is properly a resolution" (Wilen, 1971).

enriched parent compounds or metabolites. Also, the DDT-dehydrochlorinase enzyme system from the house fly and/or mosquito could be investigated for a partial resolution of *o,p'*-DDT. In most cases enzymatic or biological optical resolutions suffer from two disadvantages : the resolution may not be complete and therefore give low optical purity only; and one optical isomer only would be isolated if an appropriate biological system could be utilized.

The *o,p'*-DDT molecule does not contain a reactive functional group or "handle." Therefore neither resolution by kinetic methods, utilizing enantiomeric differences in reactions with chiral reagents (Boyle, 1971), nor chromatographic resolutions, utilizing differential diastereomer formation on a stationary substrate (Karger, 1967; Lochmüller and Souter, 1975), could be used. A stereospecific synthetic route involving an optically active precursor was not feasible since the condensation reaction giving rise to DDT creates the asymmetry of the carbon atom and chiral precursors are not involved (Adams *et al.*, 1967; Brooks, 1974). Similarly, an asymmetric synthesis[2] for this type of compound has not been described previously (Morrison and Mosher, 1971; Scott and Valentine, 1974).

Therefore, the resolution of *o,p'*-DDT was attempted via disastereomeric salt formation, the most commonly used of all resolution techniques. The literature regarding this technique was reviewed

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[2]An asymmetric synthesis is "a reaction which an achiral unit in an ensemble of substrate molecules is converted by a reactant into a chiral unit in such a manner that the stereospecific products are produced in unequal amounts" (Scott and Valentine, 1974).

by Wilen (1971) and the method typically involves the resolution of a racemic base by an optically pure acid and *vice versa* although other classes of compounds such as alcohols may be resolved similarly. The resolution of *o,p'*-DDT by this method is described below (see Materials and Methods).

This optical resolution of (±)-*o,p'*-DDT was considered important for four reasons: as a component of technical grade DDT, *o,p'*-DDT is currently being introduced into much of the world's biosphere and until its estrogenic effects and environmental fate are understood, effects on wildlife cannot be ruled out; if the enantiomers were to exhibit differing estrogenic activities this resolution would provide the opportunity for furthering the understanding of the chemistry of estrogens and their molecular steric requirements; from a strictly academic point of view this original resolution would yield further knowledge of the chemistry of DDT; lastly, the resolution route might be applicable to (±)-*o,p'*-DDD.

MATERIALS AND METHODS

CHEMICAL

The resolution of the enantiomers of *o,p'*-DDT was undertaken via the technique of diastereomeric salt formation. Since the *o,p'*-DDT molecule did not contain an active functional group to allow for a direct salt formation, it was necessary to introduce such a group to the molecule. Either an acidic or basic group could have been added to the molecule and consideration was given to both approaches.

Purification of (±)-*o,p'*-DDT

The relative expense of pure (±)-*o,p'*-DDT led to the isolation of the compound from technical grade DDT. The technical grade material was generously supplied by M. Sobelman of the Montrose Chemical Corporation of California and consisted of about 18.5% (±)-*o,p'*-DDT by gas liquid chromatographic (GLC) analysis, with the major component being *p,p'*-DDT.

The GLC used was a Varian Aerograph Model 600-D operated at 185°C with a 1.5 m x 3.2 mm O.D. pyrex glass column packed with a 1:1 mixture of 10% DC200 and 15% QF-1 on Anakrom ABS (60-80 mesh). The carrier gas was N_2 and the detector a 250 μcurie tritium source electron capture (EC) system. DDT standards were obtained from the U.S. Environmental

Protection Agency (EPA). Fractional crystallization of 9 kg of the technical grade DDT from ethanol, pentane and methanol (Haller *et al.*, 1945) led to 516.9 g (±)-*o,p'*-DDT containing about 1.3% *p,p'*-DDT. This purity compared favorably with that of supposedly 99+% pure commercial preparations of (±)-*o,p'*-DDT where GLC analyses revealed *p,p'*-DDT contamination ranging from 2.0 to 2.5% in three individual samples. No attempt was made to improve the yield of racemic *o,p'*-DDT because of the ease of preparing such large quantities. Analysis on a Perkin-Elmer 141 photoelectric polarimeter revealed that the isolated *o,p'*-DDT was racemic. The melting points reported in the Experimental section (Appendix 4) are uncorrected and the elemental analyses were carried out in the Department of Chemistry at this University. The NMR (Nuclear Magnetic Resonance) instruments used were a Varian A-60 or A-56/60 unless specified otherwise.

Monosulfonation of (±)-*o,p'*-DDT

Introduction of a sulfonic acid group to the (±)-*o,p'*-DDT molecule was carried out using a method similar to that outlined by Trojna and Hubacek (1958) for 2,2,2-trichloro-1,1-diphenylethane. The product (the sodium salt of the sulfonic acid) was very pale yellow and crystalline. Its high solubility in water, emulsion-forming properties and benzylthiouronium salt formation (Vogel, 1967) were characteristic of sulfonic acids in general. Thin layer chromatography (TLC) on silica gel G using hexane, chloroform and methanol (3:2:1 respectively) as the solvent system (recommended for polar DDT derivatives by Abou-Donia and Menzel (1968b)) gave only one migrating spot. Furthermore, an MMR spectrum revealed an increased deshielding

of the ortho proton on the *o*-Cl ring (Keith *et al.*, 1969; McKinney *et al.*, 1974) by the $-SO_3Na$ substitution which indicated that the product was 1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]benzene-4-sodium sulfonate. A sharp doublet was not obtained for this proton in the NMR spectrum, however, possibly because the salt rather than the free acid was analysed.

Attempted desulfonation of (±)-SO_3Na-*o,p'*-DDT

Although sulfonation reactions are usually reversible (desulfonation) and the ease of desulfonation varies directly with the ease of sulfonation (Morrison and Boyd, 1967) it was not possible to desulfonate the monosulfonated (±)-*o,p'*-DDT.

The first desulfonation attempts were carried out in 37% HCl at 10°C temperature increments ranging from 40-100°C. Further attempts were made in dilute (30%) and concentrated (70%) H_2SO_4 at temperatures of 80°C to the boiling points of the solutions. The latter of the attempts also involved the utilization of superheated steam (230°C).

TLC and GLC analyses revealed that no (±)-*o,p'*-DDT had been produced in any of these trials. The H_2SO_4 solutions darkened with treatment perhaps indicating further sulfonation of the DDT molecule. Dr. H. Cerfontain (personal commun.) suggested that this desulfonation probably would not be feasible in view of the difficulty in desulfonating chlorobenzene sulfonates but recommended attempting it in H_3PO_4 according to the method of Setzkorn and Carel (1963). This technique also failed to regenerate (±)-*o,p'*-DDT. It would seem that the relatively low volatility of (±)-*o,p'*-DDT (Spencer and Cliath, 1972) might have prevented it from escaping from the H_2SO_4 solution

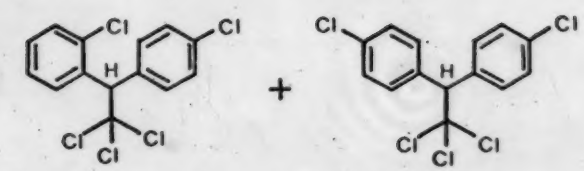
before it was resulfonated. It is possible that acidic conditions strong enough for desulfonation were obtained only in the case of the concentrated H_2SO_4 and that resulfonation under these conditions was favored also.

Mononitration of (±)-*o,p'*-DDT

The failure to introduce and remove the sulfonic acid group from the (±)-*o,p'*-DDT molecule led to the search for an alternative "handle" for salt formation. Both dinitro (Forrest *et al.*, 1946; Haller *et al.*, 1945) and mononitro derivatives of the (±)-*o,p'*-DDT were produced, reduced to their corresponding amines and coupled with optically pure acids (Appendix 4). The resolution was effected via the mononitro derivative (Figure 3).

The (±)-*o,p'*-DDT was mononitrated using a modification of the method of Sparks (1966) for chlorobenzene. The resultant reaction mixture contained five products as well as the parent compound (Figure 4). Monitoring the progress of the nitration reaction on TLC revealed that products II (1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]-4-nitrobenzene) and ii (Figure 4) were the first to appear and were followed by compound iv and finally small amounts of compounds iii and v of Figure 4. From NMR spectra the unique resonance of the ortho proton on the *o*-Cl ring of *o,p'*-DDT (Keith *et al.*, 1969; McKinney *et al.*, 1974) was an excellent indicator of the presence and position of substitutions on this ring (Figure 5). The parent compound, I, and the desired product, II, have been identified rigorously but the other structures of Figure 4 are supported by NMR spectra only and are not definitive. It would seem that the aromatic ortho, para-directing

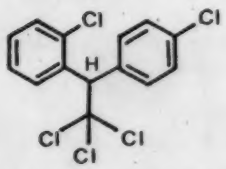
Figure 3. Route for the resolution of the enantiomers of *o,p'*-DDT via a mononitrated derivative.



~19 % (±)-o,p'- ~80 % p,p'-

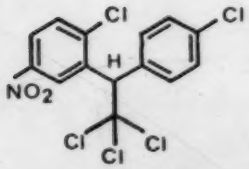
TECHNICAL GRADE DDT

↓ CRYSTALLIZATION



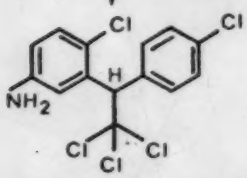
(±)-o,p'-DDT

↓ MONONITRATION

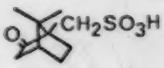


(±)-NO_2-o,p'-DDT

↓ REDUCTION



(±)-NH_2-o,p'-DDT

↓ RESOLUTION via 

(-)- AND (+)-AMINES

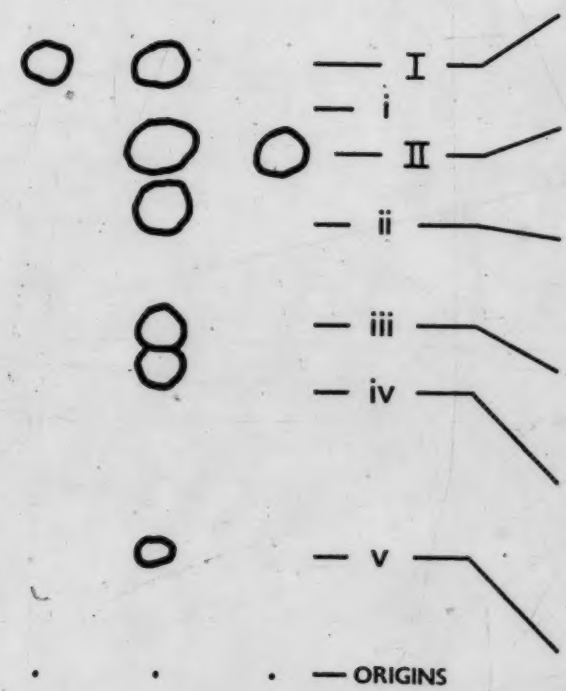
↓ DEAMINATION

(-)- AND (+)-o,p'-DDT

Figure 4. Thin layer chromatogram diagram for (a) (±)-*o,p'*-DDT (I), (b) the reaction mixture resulting from the mononitration of (±)-*o,p'*-DDT (I), and (c) (±)-NO_2-*o,p'*-DDT (II). The adsorbent was silica gel G developed with 20% diethyl ether in hexane. Compound i was an oil consisting of *o,p'*-DDT, *p,p'*-DDT plus several other compounds (GLC) and made up about 3.3% of the reaction mixture.

*See text.

**By weight (based on a 1 g test run with compounds recovered from TLC plates).



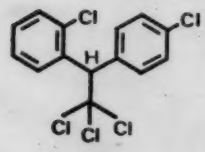
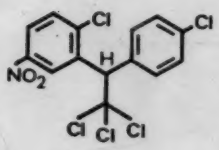
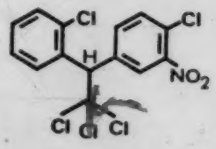
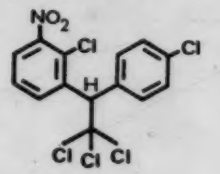
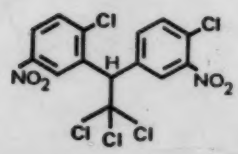
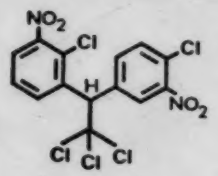
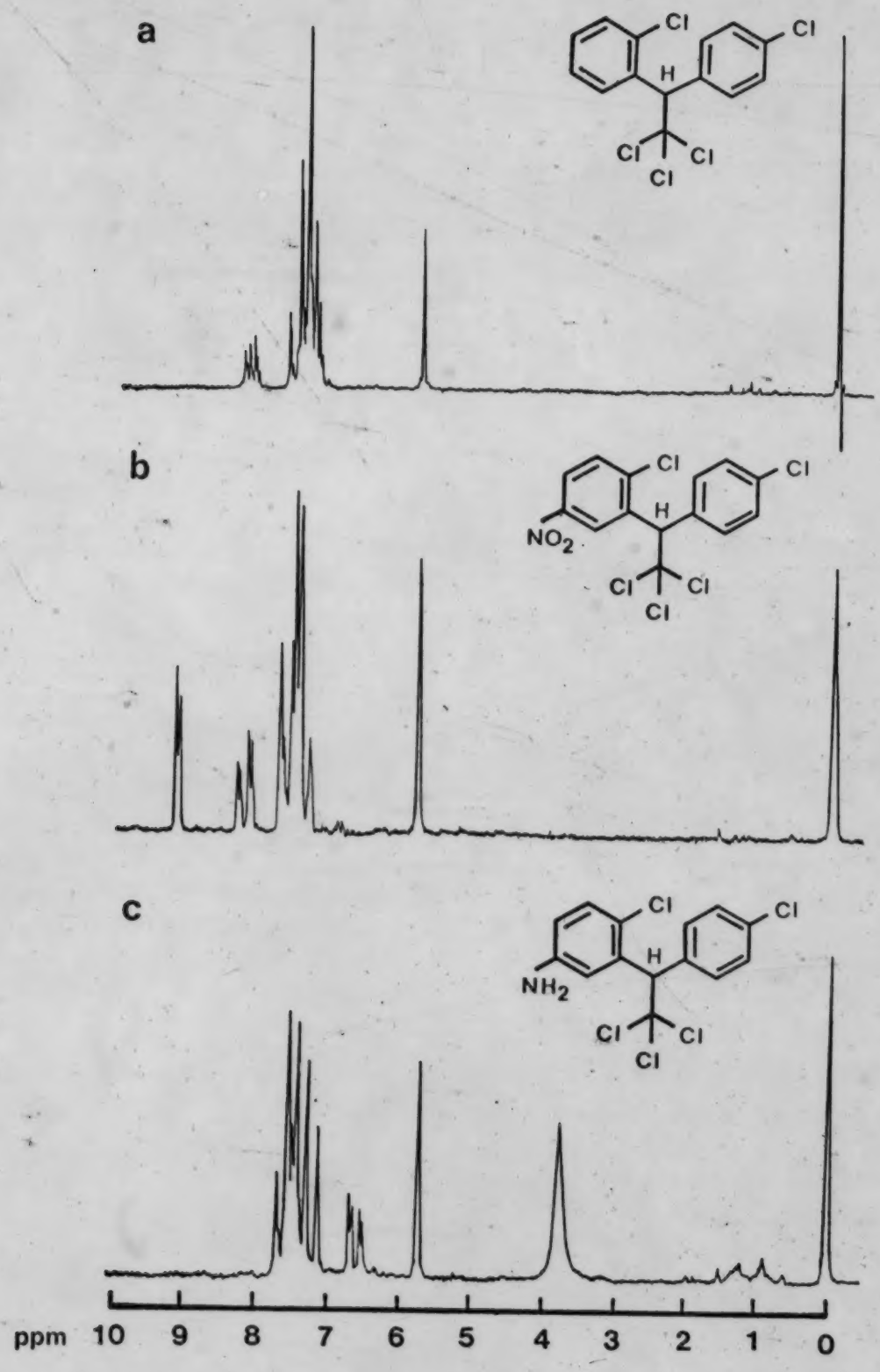
COMPOUND	PROPOSED STRUCTURE*	APPROXIMATE YIELD (%)**
I		12.4
i		54.3
II		15.0
ii		7.3
iii		10.9
iv		5.6
v		

Figure 5. NMR spectra for (a) (±)-*o,p'*-DDT (I), (b) (±)-NO_2-*o,p'*-DDT (II), and (c) (±)-NH_2-*o,p'*-DDT. See text for description. Instrumentation: Varian Anaspect EM-360 NMR spectrometer.


a
Cl
Cl
H
Cl
Cl
Cl
b
Cl
Cl
H
NO2
Cl
Cl
Cl
c
Cl
Cl
H
NH2
Cl
Cl
Cl
ppm 10 9 8 7 6 5 4 3 2 1 0

chlorines may have acted in conjunction with the meta-directing trichloroethane moiety (electron withdrawing (Abou-Donia, 1975)) to determine the sites of nitration of (±)-*o,p'*-DDT.

Stronger nitration conditions (more H_2SO_4) or additional reaction time resulted in an increased production of the dinitro compound (compound iv in Figure 4) previously described by Haller *et al.* (1945) and Forrest *et al.* (1946). Ideally the nitration reaction should have been stopped when the probability of mononitrating the *o*-Cl ring of (±)-*o,p'*-DDT to produce (±)-NO_2-*o,p'*-DDT (II) equaled the probability of mononitrating the *p*-Cl ring of (±)-NO_2-*o,p'*-DDT (II) to produce (±)-$(NO_2)_2$-*o,p'*-DDT (iv) (Figure 4). The yield of II from the nitration of four 50 g batches of I was 39.3% but 28.6% of the (±)-*o,p'*-DDT was recovered from the reaction. Obviously the 200 g of (±)-*o,p'*-DDT could have been nitrated for a longer period of time to have yielded more of the desired (±)-NO_2-*o,p'*-DDT (II) and less unaltered starting material as found for the 1 g test run of Figure 4.

Reduction of (±)-NO_2-*o,p'*-DDT

The method for the reduction of 1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]-4-nitrobenzene to its corresponding amine (1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]-4-aminobenzene) (Figures 3 and 5) was similar to that for reducing nitrobenzene to aniline (Vogel, 1967). It was, however, necessary to add ethanol for solubilization of the nitro derivative for the reduction reaction and to add $CHCl_3$ to the resulting reaction mixture in order to solubilize the amine-chlorostannate salt complex for salt hydrolysis by the NaOH. The dried ethereal extract of the (±)-amine was an oil which

could be crystallized from petroleum ether in an 87.0% yield (1 g reduction).

Diastereomeric salt formation of (±)-NH_2-*o,p'*-DDT

Because of the high yield of the reduction reaction and the ease of oxidation of exposed (±)-NH_2-*o,p'*-DDT the ethereal extract of the above reduction reaction was combined with a CH_3CN solution containing the appropriate amount of (+)-10-camphorsulfonic acid (Aldrich Chemical Co., Inc.). The quantity of acid used was a 1:1 molar ratio to the (±)-amine assuming a 100% yield of the amine from the nitro compound, presumably giving a small excess of the acid for the salt formation. Evaporation of the solvents yielded the (+)-10-camphorsulfonate salts of (-)- and (+)-NH_2-*o,p'*-DDT which were fractionally crystallized from CH_3CN or submitted to a fractional reflux technique.

Fractional crystallization of the NH_2-*o,p'*-DDT-(+)-10-camphorsulfonate salts

The initial resolution of the amine enantiomers was carried out via the classical technique of fractional crystallization of the diastereomeric salts from an appropriate solvent (Boyle, 1971; Wilen, 1971). Typically the progress of resolutions of this type is monitored by measuring changes in (1) the specific rotation of the salt being isolated, (2) the specific rotation of the amine being isolated or (3) the melting point of the salt being isolated. The resolution is considered complete when the above parameters cannot be altered further by continued treatment. As can be seen in Table 1, the specific rotations of the salts were unreliable indicators for the

Table 1. Progress of the resolution of (-)-NH_2-*o,p'*-DDT from 38.3 g (±)-NH_2-*o,p'*-DDT-(+)-10-camphorsulfonate salt by fractional crystallization from CH_3CN.

Crystallization Number	CH_3CN (ml)	Salt Yield (g)	Time (hr)	$[\alpha]_D^{RT*}$ (°) Salt	Amine
1	1250	8.5	15	0.7	- 7.1
2	120	3.8	24	3.1	- 14.9
3	120	2.0	17	---	- 30.0
4**	1000	15	45	- 43.0	- 22.9
5	500	6	11	- 62.9	- 69.3
6	340	4	24	-152.2	-119.6
7	240	1.5	40	-117.6	-168.7
8	100	0.5	66	- 63.6	-168.0

*RT = Room Temperature. For these routine rotation determinations the temperatures of the solutions were not standardized.

**Salts from the above crystallizations were recombined and seeded with (-)-amine salt with $[\alpha]_D^{RT} = -30.0°$ for the amine.

progress of the resolution of the (-)-NH_2-*o,p'*-DDT so the rotations of small amounts of the regenerated amines were used routinely to follow this preliminary resolution.

Before measuring the rotations of the amines it was essential that they be purified on a 4 cm x 1 cm O.D. acid-washed alumina column eluted with 20% diethyl ether in hexane in order to remove any brown (oxidized) material or unhydrolyzed salts.

Since crystallizations 1 to 3 of Table 1 revealed only a modest improvement of the (-)-amine rotation a small amount of (-)-amine salt ($[\alpha]_D^{RT} = -30.0^o$ for the amine) was set aside and the remaining salts recombined. Use of the -30.0^o amine-salt for seed crystals led to a relatively rapid purification of the (-)-amine salt (crystallizations 7 and 8 of Table 1).

Fractional reflux of the NH_2-*o,p'*-DDT-(+)-10-camphorsulfonate salts

A reflux technique for the separation of disastereomeric salts has been suggested by Wilen (1971) and its use in this case was regulated by qualitative rather than quantitative means. That is, because this method of resolution was so simple and effective, the amounts of salts, solvent and times of reflux to give various degrees of improvement in optical purity of the amines were not calculated.

To purify the levo amine, (±)-amine salt or salt with a predominance of (-)-amine was dissolved in boiling CH_3CN. The volume of CH_3CN was lowered by distillation until salt was seen precipitating. At this point the distillation was stopped as a large amount of salt would precipitate while the CH_3CN continued to reflux. A small volume of CH_3CN was added to the reflux flask and the solution was kept under

reflux for 2-4 hours. Larger volumes of CH_3CN added to the flask (but not giving complete dissolution of the precipitated salt) and/or longer reflux times improved the resolution of the (-)-amine. Three such serial reflux treatments produced a salt with an (-)-amine rotation of $[\alpha]_D^{25} = -170.1^{\circ}$, unimproved by further reflux, similar in rotation to the (-)-amine previously isolated above by the classical crystallization procedure.

The use of a double withdrawal crystallization technique yielded salts with little or no improvement of enantiomeric purity except, of course, in the case of the primary crystallization presented in Table 1. That is, all other isolated salts contained amines exhibiting low dextro rotations indicating that the (-)-amine salts could not be crystallized from salt solutions containing an excess of the (+)-amine. To purify the (+)-amine, (+)-amine-enriched salts recovered from the reflux mother liquors were dissolved in boiling CH_3CN. The volume of CH_3CN was reduced by distillation until a large amount of salt precipitated. This salt was recovered without further treatment and revealed an improved (+)-amine rotation. Six such treatments produced almost pure (+)-NH_2-*o,p'*-DDT. Furthermore it was found that during this resolution the increasing melting points of the salts were excellent indices of improvements in the enantiomeric purity of the amines.

Lanthanide shift reagent

An attempt was made to determine the optical purity of the resolved amines utilizing a lanthanide shift reagent, Sievers' Reagent or $Eu(fod)_3$ (Norell Chemical Co.). By use of Varian Anaspect EM-360 (60 MHz) and Perkin-Elmer R-32 (90 MHz) NMR spectrometers it was

hoped that this reagent might split the signal for the benzylic proton of racemic NH_2-*o,p'*-DDT. To 50 mg (±)-NH_2-*o,p'*-DDT in 0.4 ml CCl_4 the $Eu(fod)_3$ was added in 10 mg increments and later 100 mg increments up to a total of 980 mg of the reagent. While large shifts occurred for all proton signals no observable split in the benzylic proton signal occurred possibly because of the distance between the amine group and the asymmetric center of this molecule.

Deamination of (-)- and (+)-NH_2-*o,p'*-DDT to (-)- and (+)-*o,p'*-DDT

The resolved amines were deaminated by treating their diazonium salts with hypophosphorous acid (Sagar *et al.*, 1972). The reaction solution was allowed to stand 72 hr by which time it had cleared and the *o,p'*-DDT enantiomers could be extracted with pentane. The specific rotations of the resolved enantiomers of *o,p'*-DDT were $[\alpha]_D^{25} = -17.9^o$ and 17.7^o.

Absolute configuration of the enantiomers of *o,p'*-DDT

X-ray crystallographic analysis of (-)-*o,p'*-DDT has revealed that it possesses the R configuration (Smith, Dr. R. A.; personal commun.).

Production of (±)-OH-*o,p'*-DDT from (±)-NO_2-*o,p'*-DDT

The (±)-NO_2-*o,p'*-DDT was converted via the (±)-NH_2-*o,p'*-DDT to its diazonium salt using the method described for the deamination above. Treatment of this salt with hot H_2SO_4 yielded a reaction mixture which was extracted with diethyl ether and the extract was purified by elution from alumina and silicic acid columns. The

product consisted of about 32% OH-*o,p'*-DDE (1-chloro-2-[2,2-dichloro-1-(4-chlorophenyl)ethenyl]-4-hydroxybenzene) and about 65% (±)-OH-*o,p'*-DDT(1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]-4-hydroxybenzene determined by NMR and GLC-MS (gas liquid chromatography-mass spectrometry). The instruments used were a Varian Anaspect EM-360 NMR spectrometer and a Hewlett-Packard Model 5710A gas chromatograph coupled with a Hewlett-Packard 5980A mass spectrometer. The hydroxy derivatives were separated as their TFA (trifluoroacetyl) derivatives on a GLC column of 1/2% OV-101 on Chromosorb 750 at 185°C. The parent ion, base peak and fragmentation patterns of the MS were in agreement with both the data supplied for similar hydroxy and/or methoxy derivatives of (±)-*o,p'*-DDT and *o,p'*-DDE (Feil *et al.*, 1973; 1975) as well as the parent compounds (Sphon and Damico, 1970).

The dehydrochlorination of the (±)-*o,p'*-DDT derivative(s) to the *o,p'*-DDE derivative(s) was unexpected in the acidic medium of the reaction but the high temperature alone may have been responsible for this side reaction. A suitable solvent for crystallization of the (±)-OH-*o,p'*-DDT was not found so the product also contained an estimated 2-3% of a yellow-colored contaminant(s).

Production of (±)-*o,p'*-DDA from *o,p'*-DDE or (±)-*o,p'*-DDT

The (±)-*o,p'*-DDA was produced from *o,p'*-DDE and (±)-*o,p'*-DDT by modifying the method of Grummitt *et al.* (1946) for the preparation of *p,p'*-DDA. The concentration of KOH was raised to the same relative amount used by Gätzi and Stammbach (1946) when producing *p,p'*-DDA and *o,p'*-DDA. The yield of 47.9% (±)-*o,p'*-DDA compared favorably with the yields produced by Cristol and Haller (1945) (13%

crude product), Gätzi and Stammbach (1946) (43.0% pure product, mp 106-107°C) and Zee-Cheng and Cheng (1962) (15% crude product). The NMR spectrum had the expected similarities to that of *p,p'*-DDA (4-chloro-α-(4-chlorophenyl)benzeneacetic acid) (Keith *et al.*, 1969).

Diastereomeric salt formation of (±)-*o,p'*-DDA

The (±)-*o,p'*-DDA-(-)-α-phenylethylamine salts (α-PEA from Aldrich Chemical Co., Inc.,) have been crystallized from an ethanol-water (1:1) solvent system. Heating of this salt to dry it and evaporation to reduce the volumes of mother liquors must be avoided since the salt is apparently somewhat unstable. This discovery has invalidated the recorded rotations for crystallized *o,p'*-DDA-(-)-α-phenylethylamine salts. These rotations were small and may not have been real since they were levo, as was the α-PEA. The rotation obtained for one sample of *o,p'*-DDA crystallized from benzene following regeneration from the crystallized salt was $[\alpha]_D^{RT} = 0.76^o$ indicating that a partial resolution had taken place. It is encouraging to have found a solvent giving crystal formation and this system may allow for a straightforward resolution of (±)-*o,p'*-DDA.

BIOLOGICAL

Female Rats

Estrogenic effects of *o,p'*-DDT

For the initial study of the estrogenic effects of the *o,p'*-DDT (McBlain *et al.*, 1976) (Table 2) a total of 149 weanling (20-21 day

Table 2. Treatment groups used for comparison of the estrogenic effects of the enantiomers of *o,p'*-DDT 18 hr following a single ip injection in immature female rats.

Number of rats (N)	Compound administered ip	Dose (mg/kg)
33	---	---
10	(+)-17β-estradiol	0.11
10	(±)-*o,p'*-DDT	25
10	(±)-*o,p'*-DDT	50
10	(-)-*o,p'*-DDT	50
10	(+)-*o,p'*-DDT	50
10	(±)-*o,p'*-DDT	100
10	(-)-*o,p'*-DDT	100
10	(+)-*o,p'*-DDT	100
10	(±)-*o,p'*-DDT	150
10	(±)-*o,p'*-DDT	225
8	(-)-*o,p'*-DDT	225
8	(+)-*o,p'*-DDT	225

old; 35-50 g) female rats of the Sprague-Dawley strain were obtained as available from BioScience Animal Services of the University of Alberta. The rats were selected randomly, weighed and given a single ip injection of the appropriate test solution. The tested compounds, (±)-*o,p'*-DDT, (-)-*o,p'*-DDT, (+)-*o,p'*-DDT and (+)-17β-estradiol were dissolved in olive oil (Empress Foods Ltd.) at the solution concentration necessary to give the desired dosage (in mg per kg body weight) in 0.4 ml of solvent for a 45.0 g rat. The injected volumes were adjusted further according to the individual rat weights to give the correct dose in mg/kg. The mean weight of the rats injected was 42.4 g. The (+)-17β-estradiol was supplied by the Sigma Chemical Co. and the (±)-*o,p'*-DDT by the Aldrich Chemical Co., Inc. while the enantiomers of *o,p'*-DDT were prepared as described previously (McBlain and Wolfe, 1975). The (+)-17β-estradiol solution used was a dilution of a stock solution prepared by dissolving 0.1250 g of the estradiol in 5 ml of ethanol and diluting the solution to 100 ml with the olive oil giving a 1.25 mg/ml solution. The concentration of this stock solution was verified by diluting it 1:100 and measuring the uv absorption (λ=280mμ) on a Beckman DB-G spectrophotometer.

The injected rats were placed in individual wire mesh-topped polypropylene cages (28.5 x 17.5 x 12 cm) with food (Wayne Lab-Blox, Allied Mills Inc. or Teklad mouse/rat diet) and water supplied *ad libitum*. The temperature in the experimental room was maintained at 22 ± 2°C. Eighteen hours following the injection the rats were weighed and sacrificed by cervical dislocation. Whole uteri plus anterior vaginal tissues were excised and the uterine horns were severed from the vagina immediately posterior to the cervices while the horns were

separated by a 45° angle. Enough tissue was left at the junction of the uterine horns so that they just remained joined and they were stripped of mesentery, blotted dry, weighed to the nearest 0.1 mg on a Mettler analytical balance and placed in a culture tube containing 1.0 ml 30% KOH. Uterine glycogen levels were determined by the method of Seifter *et al.* (1950) utilizing the Na_2SO_4 and temperature recommendations of Van Handel (1965). That is, after the addition of 1.25 ml of ethanol to the sample, 0.125 ml of a saturated Na_2SO_4 solution was added to aid in the precipitation of the glycogen. Following the addition of the anthrone reagent the color was developed by heating at 90°C for 20 minutes.

Estrogenic effects of *o,p'*-DDT derivatives

Several derivatives of *o,p'*-DDT were tested similarly for their relative estrogenic activities as outlined in Table 3. Two relatively inactive compounds, (±)-NO_2-*o,p'*-DDT and (±)-*o,p'*-DDA, were tested at 8 mg/rat, the screening dose employed by Bitman and Cecil (1970). The other compounds were administered using the preferred mg/kg dose levels as above. The sc route was investigated for (+)-17β-estradiol to determine if the submaximal ip response reported by McBlain *et al.* (1976) for this compound (using the same solution concentration) could be related to the route of administration. Furthermore, a time-response study using 5 rats per group at 100 mg/kg of (±)-*o,p'*-DDT was carried out to ascertain whether the individual variation within the results reported in the previous study (McBlain *et al.*, 1976) could be related to the time required to obtain the uterine responses.

The rats were caged as above and the uterine parameters, wet

Table 3. Treatment groups used for comparison of the estrogenic effects of various *o,p'*-DDT derivatives in immature female rats.

Number of rats (N)	Compound administered	Dose		Time of sacrifice (hr)	Route
8	(±)-NO_2-*o,p'*-DDT*	8	mg/rat	18	ip
9	(±)-*o,p'*-DDA*	8	mg/rat	18	ip
8	(±)-OH-*o,p'*-DDT (65%) plus OH-*o,p'*-DDE (32%)*	25	mg/kg	18	ip
6	(±)-SO_3Na-*o,p'*-DDT*	25	mg/kg	18	ip
8	(±)-NH_2-*o,p'*-DDT*	25	mg/kg	18	ip
6	(-)-NH_2-*o,p'*-DDT*	1	mg/kg	18	ip
6	(+)-NH_2-*o,p'*-DDT*	1	mg/kg	18	ip
6	(+)-17β-estradiol	0.11	mg/kg	18	sc
5	(±)-*o,p'*-DDT	100	mg/kg	12	ip
5	(±)-*o,p'*-DDT	100	mg/kg	16	ip
5	(±)-*o,p'*-DDT	100	mg/kg	20	ip
5	(±)-*o,p'*-DDT	100	mg/kg	24	ip

*The preparation of these compounds is described above.

weight and glycogen content, were measured in the manner described previously. For both rat studies and most of the quail studies below the data were subjected to an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test for significant F statistics (Sokal and Rohlf, 1969).

Female Japanese quail

Estrogenic effects of *o,p'*-DDT by the ip route

A total of 58 immature (26-29 days old; 70-93 g) female Japanese quail were obtained from BioScience Animal Services of the University of Alberta. The birds were weighed, banded and randomly distributed among the five experimental groups of Table 4. Groups of 10-15 birds were placed in steel wire cages (55 x 32 x 31 cm) and given food (turkey starter; Federated Co-operatives, Ltd.) and water *ad libitum*. The temperature of the experimental room was maintained at 19-21°C and the light regime was 8L:16D (8 hours of light to 16 hours of dark/24 hours), a photoperiod known to be non-stimulatory for sexual maturation (Tanaka *et al.*, 1965; Wilson *et al.*, 1962).

The tested compounds, (+)-17β-estradiol, (±)-*o,p'*-DDT, (-)-*o,p'*-DDT and (+)-*o,p'*-DDT (described above) were dissolved in an appropriate volume of 5% ethanol in olive oil (Nabob Foods) to give the desired dose in 0.4 ml of solution (Table 4). Once a day for three days the birds were given single ip injections of the prescribed test solution and 72 hours after the first injection a blood sample was taken by cardiac puncture and the birds sacrificed by cervical dislocation.

The oviducts were excised, trimmed of mesentery, weighed to the

nearest 0.1 mg and placed in 1 ml 30% KOH. From certain of the birds the livers, adrenals and ovaries were removed and their wet weights measured. Oviducal glycogen determinations were carried out as described for the rat uteri above. The blood samples were centrifuged at 2100 rpm for 15 minutes and the serum frozen for later analyses. For the calcium analyses the serum was thawed, mixed on a vortex mixer and diluted 1:100 by delivering 50 µl of serum into 5 ml of a 1% KCl solution. The calcium standards were 0.5, 1.0 and 3.0 ppm dilutions of a 1000 ppm stock solution made up by dissolving 2.4973 g of $CaCO_3$ (heated 13 hr at 150°C) in 300 ml of double distilled water, adding 10 ml 37% HCl and diluting to 1 litre (Dean and Rains, 1971). The serum calcium levels were determined by flame emission spectrometry using a Jarrell-Ash JA 82-270 Atomsorb atomic absorption-flame emission spectrophotometer.

Table 4. Treatment groups used for the comparison of the estrogenic effects of the enantiomers of *o,p'*-DDT by three daily ip injections in immature female Japanese quail.

Number of Birds (N)	Compound administered ip	Dose (mg/day)	Solution concentration (mg/ml)
10	---	---	---
10	(+)-17β-estradiol	0.05	0.125
13	(±)-*o,p'*-DDT	5	12.5
13	(-)-*o,p'*-DDT	5	12.5
12	(+)-*o,p'*-DDT	5	12.5

Estrogenic effects of (±)-*o,p'*-DDT by the oral route-feed

A second group of 26 immature female quail (29 days old; 44-77 g) were weighed, banded and divided randomly into two groups of 6 and two groups of 7 birds (Table 5).

Table 5. Treatment groups used in the test for estrogenic effects of 200 ppm of (±)-*o,p'*-DDT in the feed of immature female Japanese quail.

Number of birds (N)	Compound administered	Dose	Treatment period (days)
6	---	---	3
6	(±)-*o,p'*-DDT	200 ppm in the feed	3
7	---	---	7
7	(±)-*o,p'*-DDT	200 ppm in the feed	7

One group of 6 birds and one of 7 were treated with 200 ppm of (±)-*o,p'*-DDT in the feed. The feed was prepared by adding 0.22 g of (±)-*o,p'*-DDT in 100 ml acetone to 1 kg of feed (Federated Co-operatives turkey starter) in a 4 l beaker. A 10% excess of (±)-*o,p'*-DDT was added to allow for any loss of the DDT on the beaker sides or foil. The acetone-wetted feed was mixed with a glass stir rod and poured onto aluminum foil in a fume hood for drying. A further 180 ml of acetone was used to rinse the beaker sides onto the feed and later to rinse the foil surface onto the feed. GLC analysis of 1 g of the feed (Appendix 5) obtained from an 18 g sample ground in a mortar revealed 255 ppm of *o,p'*-DDT present. This indicated that the 10% excess of (±)-*o,p'*-DDT

added to the feed may not have been lost and/or that the feed had not been mixed adequately. Control feed was treated similarly with acetone only and was found to contain less than 0.5 ppm of total DDT residues. The control and (±)-*o,p'*-DDT-treated groups each consumed almost all of the 1 kg of their prepared feed during the study.

On the appropriate day the birds were sacrificed by $CHCl_3$ anaesthesia and a blood sample taken from the heart. The oviducts were excised, weighed and their lengths measured. The livers and blood were treated as in the ip injection study above.

Estrogenic effects of (-)- and (+)-*o,p'*-DDT by the oral route-capsules

A third group of 6 immature female quail (29 days old; 65-77 g) were treated twice daily with gelatin capsules (size 5; Eli Lilly and Co.) containing either (-)-*o,p'*-DDT or (+)-*o,p'*-DDT at the doses shown in Table 6. Following the three days of treatment (72 hours after the first capsule) the birds were sacrificed by $CHCl_3$ anaesthesia and blood samples were removed and handled as above. Furthermore, a sample of subcutaneous fat was removed from the area adjacent to the apex of the furculum and along with the uropygial gland was frozen for later analysis.

The right lobes of the uropygial glands were laid open by several longitudinal and transverse scalpel cuts. These samples and the fat samples were cleaned up (Appendix 6) and subjected to GLC analysis for the residues of *o,p'*-DDT present.

Table 6. Treatment groups and doses for the enantiomers of *o,p'*-DDT administered via the oral route in capsules to immature female Japanese quail.

Number of birds (N)	Compound administered	Dose (mg)						
		Capsule number						Total
		1*	2*	3	4	5*	6	
3	(-)-*o,p'*-DDT	14.0	10.3	8.9	7.6	10.6	6.0	57.4
		16.1	8.2	8.9	8.1	9.9	6.0	57.2
		12.1	11.2	9.1	8.8	10.1	7.2	58.2
3	(+)-*o,p'*-DDT	13.6	11.0	7.9	6.5	13.6	4.7	57.3
		14.4	8.6	8.8	7.4	12.2	5.7	57.1
		17.2	11.9	9.7	7.6	14.9	5.9	67.2

*Administered after ethanol added to capsule.

Male Japanese quail

Estrogenic effects of *o,p'*-DDT by the sc route

Two groups of male quail were used to test the effectiveness of the enantiomers of *o,p'*-DDT as estrogenic agents in a male system. The first group of males was used in a preliminary study designed to determine what dose of (+)-17β-estradiol would be necessary to exhibit estrogenic activity in the male birds under the conditions used.

Thirty-four 56 day old males (64-101 g) which had been kept on an 8L:16D light regime from 18 days of age (12L:12D previously) were divided randomly into seven groups of 4 and two groups of 3 as in Table 7.

One group of 4 was sacrificed immediately and another group of 4

Table 7. Treatment groups used for the determination of the estrogenic activity of sc injections of (+)-17β-estradiol or the enantiomers of *o,p'*-DDT in male Japanese quail switched from a short to a long photoperiod.

Number of birds (N)	Compound administered sc	Dose/2 days (mg)	Light regime
4	---	---	---
4	---	---	8L:16D
4	---	---	14L:10D
4	(+)-17β-estradiol	0.0005	14L:10D
3	(+)-17β-estradiol	0.0005	14L:10D
3	(+)-17β-estradiol	0.05	14L:10D
4	(+)-17β-estradiol	0.5	14L:10D
4	(-)-*o,p'*-DDT	5	14L:10D
4	(+)-*o,p'*-DDT	5	14L:10D

left on the 8L:16D lighting. All other groups were switched to 14L:10D. These light regimes were selected since it has been shown that the 24 hour ratio of 12L:12D and 24 hour light regimes with shorter light periods have little stimulatory effect on the testes development of young quail (Tanaka *et al.*, 1965). The 24 hour light regimes of 14 or more hours of light are stimulatory. The birds were caged similar to the females above and given food and water *ad libitum*. The temperature in the short daylength room was 17.5-18.5°C and in the long daylength room 21.5-23.0°C. Every second day all test birds received sc injections of 0.4 ml of 5% ethanol in olive oil containing no solute or 0.0005 mg, 0.005 mg, 0.05 mg or 0.5 mg of (+)-17β-estradiol. The estradiol solutions used were dilutions of the stock solution described above. Two

other groups received 5 mg of either (-)-*o,p'*-DDT or (+)-*o,p'*-DDT in 0.4 ml olive oil only. At 66 days of age (10 days after the first injection) a blood sample was removed by cardiac puncture and the birds sacrificed by cervical dislocation. The testes were excised, trimmed and weighed to the nearest 0.1 mg and immediately placed in Bouin's solution.

Estrogenic effects of *o,p'*-DDT by the ip route

A second group of 70 male quail (96-133 g) was treated similar to the above group of Table 7 except that the injections used were ip and two doses of the estradiol and two doses of (±)-*o,p'*-DDT were used as shown in Table 8. For this study the *o,p'*-DDT compounds were dissolved in 5% ethanol in olive oil.

As above, the birds were injected every other day and 10 days after the first injection were sacrificed by cervical dislocation. Blood and testes samples were treated as before. From certain of the birds the livers, adrenals and spleens were excised and weighed. Selected serum calcium determinations were carried out as above. Some of the testes were fixed in Bouin's solution, imbedded in paraffin, sectioned at 7μ and stained in iron hemotoxylin and eosin.

Table 8. Treatment groups used for the determination of the estrogenic activity of ip injections of (+)-17β-estradiol or the enantiomers of *o,p'*-DDT in male Japanese quail switched from a short to a long photoperiod.

Number of birds (N)	Compound administered ip	Dose/2 days (mg)	Light regime
11	---	---	---
8	---	---	8L:16D
9	---	---	14L:10D
6	(+)-17β-estradiol	0.05	14L:10D
8	(+)-17β-estradiol	0.5	14L:10D
7	(±)-*o,p'*-DDT	5	14L:10D
6	(±)-*o,p'*-DDT	10	14L:10D
8	(-)-*o,p'*-DDT	5	14L:10D
7	(+)-*o,p'*-DDT	5	14L:10D

RESULTS

Female rats

Initial body weights

The mean initial body weights (body weights immediately prior to injection) for the 25 treatments groups of rats are presented in Tables 9 and 10. In Table 9 the data for the 13 groups of the first part of the study (Table 2) were compared at $p \leq .01$ and $p \leq .05$ and the group injected with 225 mg/kg of (+)-*o,p'*-DDT was significantly lighter than the control group at $p \leq .05$. No other groups differed from the controls.

If the data for the 13 groups of Table 2 were combined with the data for the 12 groups of the second part of the study (Table 3) as shown in Table 10, no groups differed from the control for the parameter of mean initial body weights at either the 1 or 5% levels. This combination of the data for the first 13 groups with the data for the latter 12 groups changed the statistical results mainly because of the smaller sample sizes used in the latter 12 groups. This added some bias to the statistical treatment of the data by necessitating larger differences among the group means in order to render the differences statistically significant.

It would appear unlikely that the initial weights of the injected

Table 9. Mean initial body weights for the treatment groups used for comparison of the estrogenic effects of the enantiomers of *o,p'*-DDT in immature female rats.

	Mean initial body weights (g) ±SEM												
	Treatment groups*												
	(+)	(-)	(+)	(+)		(±)		(-)	(±)	(±)	(±)	(±)	(-)
	225	50	100	50	E	100	C	100	225	150	50	25	225
N	8	10	10	10	10	10	33	10	8	10	10	10	10
	39.5	41.8	42.3	42.5	42.6	42.6	43.1	43.5	43.5	43.8	44.1	44.3	44.4
	±1.1	±1.1	±1.2	±1.6	±0.8	±1.6	±0.6	±1.0	±1.1	±1.2	±1.1	±1.0	±0.8

$p \leq$**

.01

.05

*Bracketed sign indicates enantiomeric or racemic *o,p'*-DDT. Numeral is the dose in mg/kg. C = control and E = (+)-17β-estradiol.

**Statistical treatment consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Like means are underlined.

Table 10. Mean injection body weights for the 25 treatment groups of female rats.

Duncan's group* number	N	Treatment**	Mean initial body weight (g) ±SEM	Duncan's group* number	N	Treatment**	Mean initial body weight (g) ±SEM
1	33	control	43.1±0.6	14	8	NO_2	43.7±0.8
2	10	estradiol	42.6±0.8	15	9	DDA	40.2±1.1
3	10	(±) 25	44.3±1.0	16	8	OH	43.7±1.0
4	10	(±) 50	44.1±1.1	17	6	SO_3Na	43.2±1.0
5	10	(-) 50	41.8±1.1	18	8	(±) NH_2	41.5±1.4
6	10	(+) 50	42.5±1.6	19	6	(-) NH_2	43.7±1.8
7	10	(±)100	42.6±1.6	20	6	(+) NH_2	43.8±1.8
8	10	(-)100	43.5±1.0	21	6	E sc	43.1±0.7
9	10	(+)100	42.3±1.2	22	5	(±)100 12 hr	45.5±1.3
10	10	(±)150	43.8±1.2	23	5	16 hr	43.4±1.4
11	10	(±)225	43.5±1.1	24	5	20 hr	42.0±1.7
12	8	(-)225	44.4±0.8	25	5	24 hr	43.9±2.0
13	8	(+)225	39.5±1.1				

Group	13	15	18	5	24	9	6	2	7	21	1	17	23	8	11	16	19	14	10	20	25	4	3	12	22	p≤
																										0.1
																										0.5

*Statistical treatment consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Groups with like means are underlined.

**Listed as in Table 2.

***Listed as in Table 3.

rats affected the results of this study except perhaps in the case of the (+)-*o,p'*-DDT at 225 mg/kg group if lighter rats were assumed to be less capable of responding to an estrogen. However, a second way of determining if the initial body weights could have affected the responses of the rats to the various treatments was utilized. The correlation coefficient between the mean initial body weights for each group and the mean estrogenic response of each group (for the most sensitive parameter measured, uterine glycogen) was calculated and found not to be significant ($r = 0.1417$).

18 hr body weight changes

The mean 18 hr body weight changes for the rats were recorded and compared among the treatment groups. For the 13 groups of rats of Table 11 none of the mean weight changes differed from that of the controls at $p \leq .01$ but at $p \leq .05$ both the (-)- and (+)-*o,p'*-DDT groups at 225 mg/kg differed significantly from the controls. Also from Table 11 it can be seen that there was a tendency for those groups treated with the higher doses of the *o,p'*-DDT compounds to have mean weight gains lower than the rats treated at the lower doses although the differences were not significant in most cases.

The combined results for mean 18 hr body weight changes for all 25 groups of rats are shown in Table 12. For this statistical treatment the mean weight gains of no groups differed from that of the control at $p \leq .01$ but at $p \leq .05$ the groups treated with (±)-NH_2-*o,p'*-DDT at 25 mg/kg and with (±)-*o,p'*-DDA at 8 mg/rat did differ significantly from the controls. For the treatments of the two acidic derivatives, 8 mg/rat of (±)-*o,p'*-DDA and 25 mg/kg of (±)-SO_3Na-*o,p'*-DDT, the rats

Table 11. Mean 18 hr body weight changes for the treatment groups used for comparison of the estrogenic effects of the enantiomers of *o,p'*-DDT in immature female rats.

18 hr body weight changes (g) ±SEM													
Treatment groups*													
(-)	(+)	(+)	(±)	(±)	(±)	E	C	(±)	(+)	(-)	(±)	(-)	
225	225	100	225	150	100			25	50	100	50	50	
1.8	2.0	2.6	2.7	3.3	3.6	3.6	3.7	4.1	4.3	4.4	4.7	5.3	
±.5	±.8	±.4	±.4	±.8	±.6	±.4	±.4	±.5	±.5	±.3	±.8	±.4	$p \leq$**
													0.1
													0.5

*Bracketed sign indicates enantiomeric or racemic *o,p'*-DDT. Numeral is dose in mg/kg. C = control and E = (+)-17β-estradiol.

**Statistical treatment consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Like means are underlined.

Table 12. Mean 18 hr body weight changes for the 25 treatment groups of immature female rats.

Duncan's group number*	N	Treatment**	Mean body weight change (g) ±SEM	Duncan's group number*	N	Treatment***	Mean body weight change (g) ±SEM
1	33	control	3.7±0.4	14	8	NO_2	3.3±0.3
2	10	estradiol	3.6±0.4	15	9	DDA	1.1±0.4
3	10	(±) 25	4.1±0.5	16	8	OH	4.2±0.5
4	10	(±) 50	4.7±0.8	17	6	SO_3Na	1.8±0.4
5	10	(-) 50	5.3±0.4	18	8	(±) NH_2	1.0±0.6
6	10	(+) 50	4.3±0.5	19	6	(-) NH_2	4.4±0.4
7	10	(±)100	3.6±0.6	20	6	(+) NH_2	4.4±0.3
8	10	(-)100	4.4±0.3	21	6	E sc	3.6±0.5
9	10	(+)100	2.6±0.4	22	5	(±) 100 12 hr	1.9±1.1
10	10	(±)150	3.3±0.8	23	5	16 hr	4.0±0.4
11	10	(±)225	2.7±0.4	24	5	20 hr	3.7±0.5
12	8	(-)225	1.8±0.5	25	5	24 hr	4.1±0.7
13	8	(+)225	2.0±0.8				

Duncan's test result*

Group	18	15	17	12	22	13	9	11	14	10	7	2	21	1	24	23	25	3	16	6	19	20	8	4	5	$p \leq$
																										.01
																										.05

*Statistical treatment consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Groups with like means are underlined.

**Listed as in Table 2.

***Listed as in Table 3.

exhibited extreme "discomfort" within about 2 minutes of the ip injection as evidenced by contractions of the abdominal muscles, staggering body movements and closed eyes.

It was of interest to ascertain whether the lower mean body weight gains of some groups may have resulted from the toxic effects of the treatments or whether they were merely an index of the maturity of the rats as reflected by initial body weights. Therefore the correlation coefficient between mean initial body weights and mean weight changes for the 25 groups of rats was calculated ($r = 0.2934$) and found not to be significant. While the results were not conclusive there was some evidence that higher doses or more toxic compounds may have affected the 18 hr body weight changes.

Uterine wet weights and uterine glycogen levels

The responses of the immature female rat uteri to the enantiomers and racemic modification of *o,p'*-DDT are shown in Table 13 and Figures 6, 7, and 8. The dose-response curves for the racemic compound were similar to those previously reported (Cecil *et al.*, 1971a) while the measured responses to the enantiomeric forms of *o,p'*-DDT differed significantly ($p \leq .01$) at all three dose levels studied, except for the parameter of uterine wet weight at 50 mg/kg. The levo enantiomer of *o,p'*-DDT was the more active estrogen in the immature female rats.

The statistical analyses of the uterine responses of all 25 treatment groups are shown in Tables 14, 15, and 16. These Tables should be used for statistical reference only since most of the data presented in them also appears in the more easily understood Figures 6 to 11.

Figures 9, 10, and 11 illustrate the uterine responses to several

Table 13. Effects of *o,p'*-DDT on uterine wet weight and uterine glycogen levels in immature female rats.

Mean uterine glycogen (µg/uterus) ±SEM Treatment groups*													
Control	(+)225	(+)50	(+)100	(±)25	(±)50	(-)50	E	(±)100	(±)225	(±)150	(-)100	(-)225	
23.0 ±1.1	35.9 ±1.7	37.1 ±2.3	42.7 ±2.6	42.7 ±2.7	54.6 ±5.2	71.5 ±7.0	75.4 ±4.0	77.3 ±6.8	80.5 ±3.3	84.5 ±4.7	94.3 ±5.6	104.9 ±2.7	$p^{<*}$ .01 .05

Mean uterine wet weight (mg) ±SEM Treatment groups*													
Control	(+)225	(+)100	(±)25	(+)50	(±)50	(-)50	(±)225	(±)100	E	(±)150	(-)100	(-)225	
23.7 ±0.8	26.4 ±0.8	28.7 ±1.2	29.5 ±1.8	30.8 ±1.6	31.6 ±2.2	34.5 ±2.3	37.1 ±1.3	37.2 ±1.7	37.4 ±1.5	39.2 ±2.1	42.9 ±3.3	48.4 ±1.9	.01 .05

Mean uterine glycogen concentration (µg)/uterine wet weight (mg) ±SEM Treatment groups*													
Control	(+)50	(+)225	(±)25	(+)100	(±)50	E	(-)50	(±)100	(±)150	(-)225	(±)225	(-)100	
0.97 ±.04	1.20 ±.05	1.36 ±.05	1.46 ±.07	1.49 ±.07	1.71 ±.07	2.03 ±.09	2.06 ±.14	2.07 ±.15	2.17 ±.09	2.18 ±.08	2.19 ±.10	2.23 ±.08	.01 .05

*Bracketed sign indicates enantiomeric or racemic *o,p'*-DDT. E = (+)-17β-estradiol. Numeral indicates dose in mg/kg. Statistical treatment consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Like means are underlined.

Figure 6. Dose-response relationship for the effect of (±)-*o,p'*-DDT on uterine glycogen levels (µg/uterus) of immature female rats 18 hr after a single ip injection. The effects of one dose level of (+)-17β-estradiol and three dose levels of (-)- or (+)-*o,p'*-DDT are shown also. Each point represents a mean ± its 95% confidence limits where N = 10 for all groups except the control (N = 33) and (-)- or (+)-*o,p'*-DDT at 225 mg/kg (N = 8) groups.

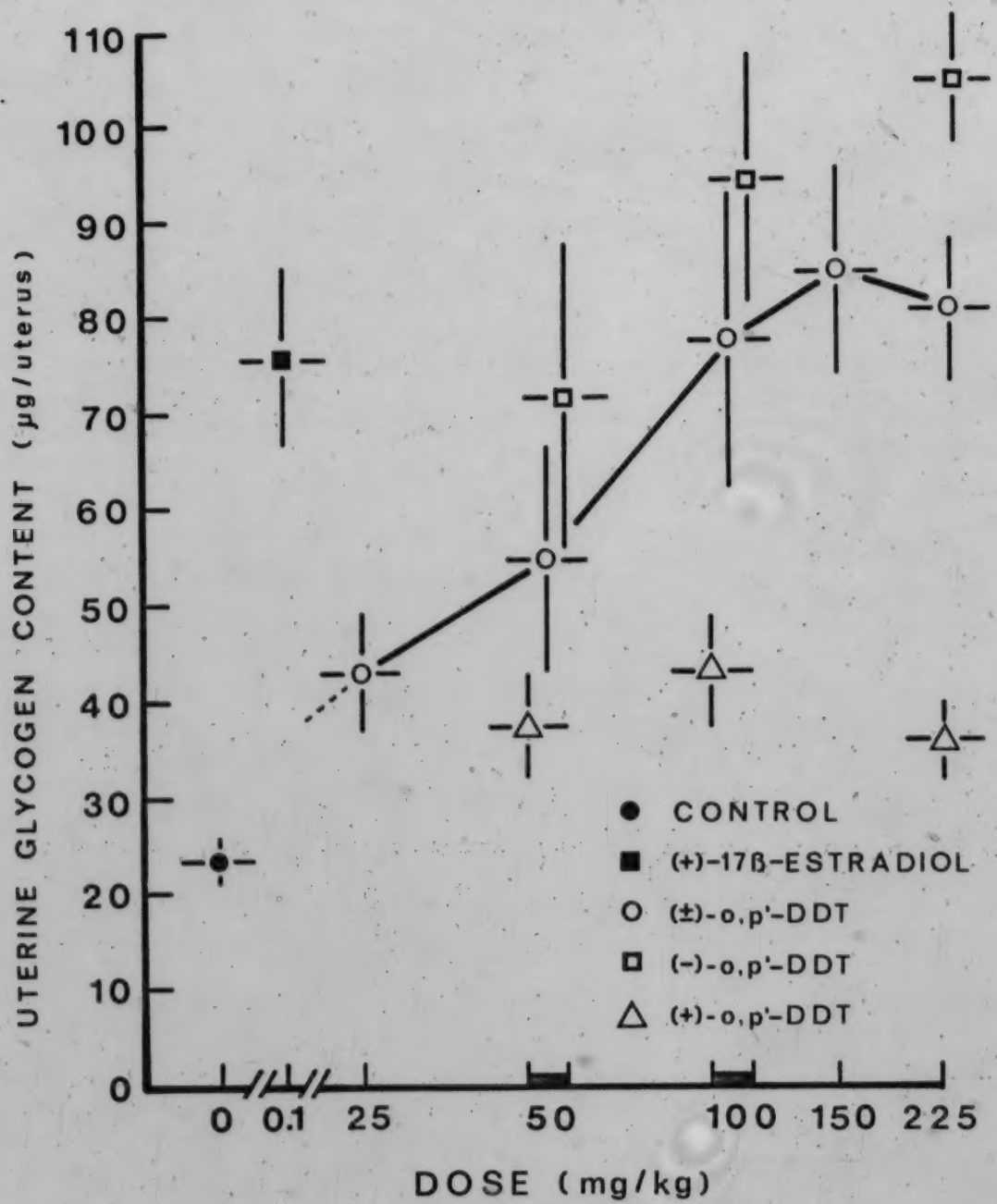

UTERINE GLYCOGEN CONTENT (µg/uterus)
110
100
90
80
70
60
50
40
30
20
10
0
0 0.1 25 50 100 150 225
DOSE (mg/kg)
CONTROL
(+)-17ß-ESTRADIOL
(±)-o,p'-DDT
(-)-o,p'-DDT
(+)-o,p'-DDT

Figure 7. Dose-response relationship for the effect of (±)-*o,p'*-DDT on uterine wet weights (mg) of immature female rats 18 hr after a single ip injection. The effects of one dose level of (+)-17β-estradiol and three dose levels of (-)- and (+)-*o,p'*-DDT are shown also. Each point represents a mean ± its 95% confidence limits where N = 10 for all groups except the control (N = 33) and (-)- and (+)-*o,p'*-DDT at 225 mg/kg (N = 8) groups.

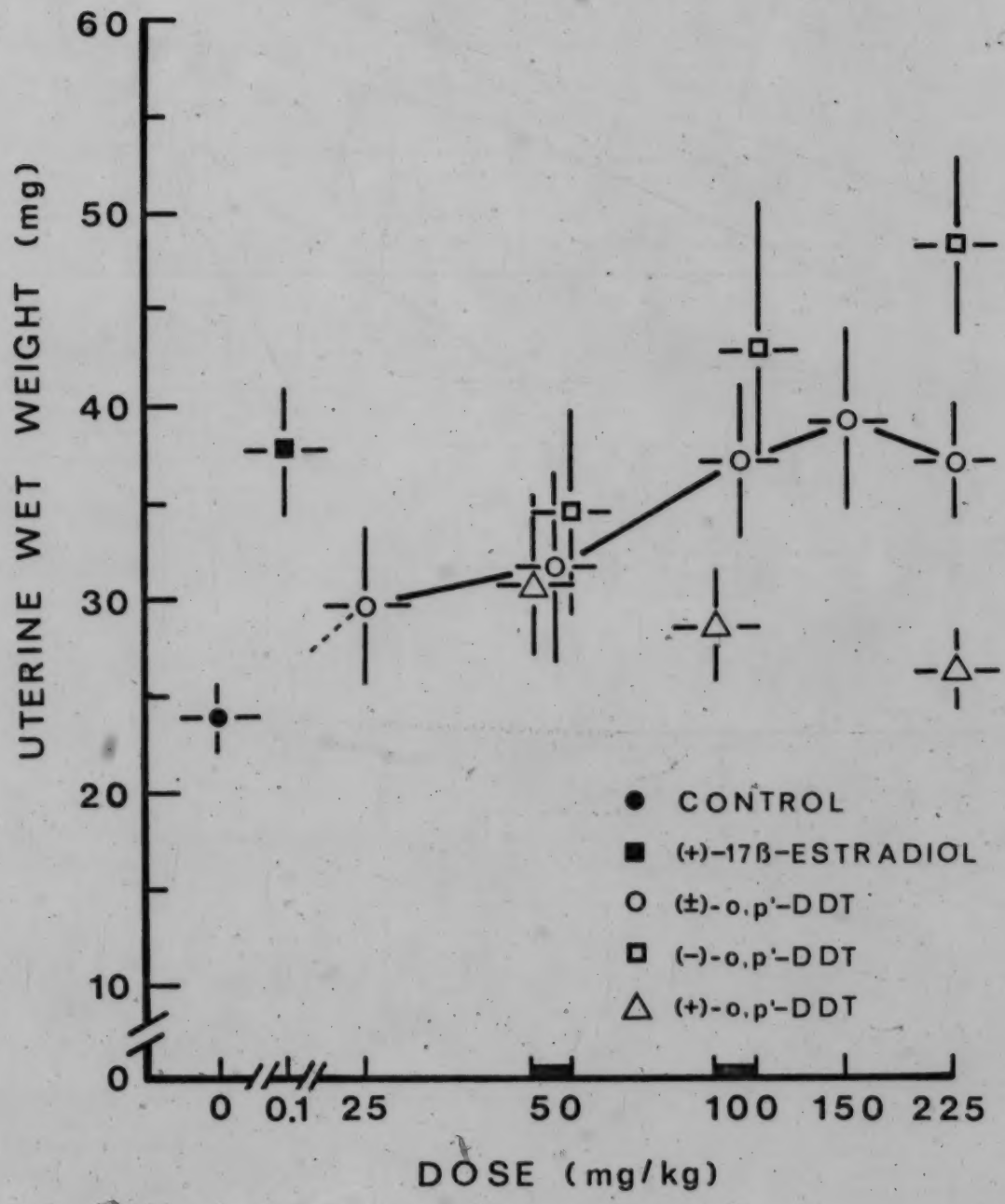

UTERINE WET WEIGHT (mg)
60
50
40
30
20
10
0
0 0.1 25 50 100 150 225
DOSE (mg/kg)
CONTROL
(+)-17ß-ESTRADIOL
(±)-o,p'-DDT
(-)-o,p'-DDT
(+)-o,p'-DDT

Figure 8. Dose-response relationship for the effect of (±)-*o,p'*-DDT on uterine glycogen concentration (µg glycogen/mg uterus) of immature female rats 18 hr after a single ip injection. The effects of one dose level of (+)-17β-estradiol and three dose levels of (-)- or (+)-*o,p'*-DDT are shown also. Each point represents a mean ± its 95% confidence limits where N = 10 for all groups except the control (N = 33) and (-)- and (+)-*o,p'*-DDT at 225 mg/kg (N = 8) groups.

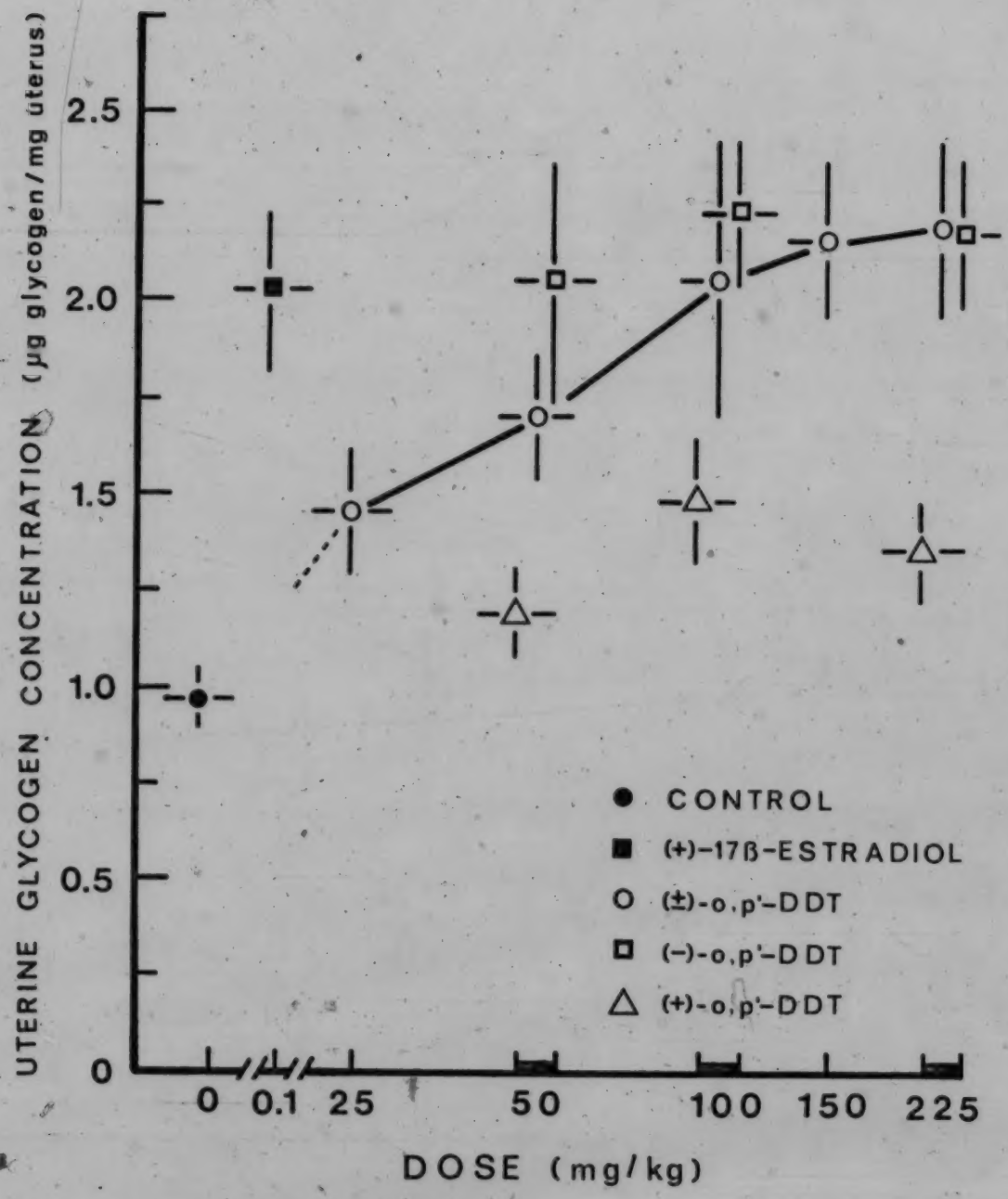

UTERINE GLYCOGEN CONCENTRATION (µg glycogen/mg uterus)
2.5
2.0
1.5
1.0
0.5
0
0 0.1 25 50 100 150 225
DOSE (mg/kg)
CONTROL
(+)-17ß-ESTRADIOL
(±)-o,p'-DDT
(-)-o,p'-DDT
(+)-o,p'-DDT

Table 14. Mean uterine glycogen levels for the 25 treatment groups of immature female rats.

Duncan's group number*	N	Treatment**	Mean uterine glycogen (µg/uterus) ±SEM	Duncan's group number*	N	Treatment***	Mean uterine glycogen (µg/uterus) ±SEM
1	33	control	23.0±1.1	14	8	NO_2 8 mg/rat	52.5± 8.8
2	10	estradiol	75.4±4.0	15	9	DDA 8 mg/rat	45.4± 4.9
3	10	(±) 25	42.7±2.7	16	8	OH 25 mg/kg	66.9± 6.5
4	10	(±) 50	54.6±5.2	17	6	SO_3Na 25 mg/kg	24.2± 3.1
5	10	(-) 50	71.5±7.0	18	8	(±) NH_2 25 mg/kg	105.3± 4.1
6	10	(+) 50	37.1±2.3	19	6	(-) NH_2 1 mg/kg	64.9±10.7
7	10	(±)100	77.3±6.8	20	6	(+) NH_2 1 mg/kg	26.9± 4.2
8	10	(-)100	94.3±5.6	21	6	E sc .1 mg/kg	109.1±13.6
9	10	(+)100	42.7±2.6	22	5	(±)100 mg/kg 12 hr	58.9± 5.2
10	10	(±)150	84.5±4.7	23	5	16 hr	76.5± 2.2
11	10	(±)225	80.5±3.3	24	5	20 hr	63.2± 8.7
12	8	(-)225	104.9±2.7	25	5	24 hr	66.5± 2.6
13	8	(+)225	35.9±1.7				

Duncan's test result*

Group	1	17	20	13	6	9	3	15	14	4	22	24	19	25	16	5	2	23	7	11	10	8	12	18	21	p≤
																										.01
																										.05

*Statistical treatment consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Groups with like means are underlined.

**Listed as in Table 2.

***Listed as in Table 3.

Table 15. Mean uterine wet weights for the 25 treatment groups of immature female rats.

Duncan's group number*	N	Treatment**	Mean uterine wet weight (mg) ±SEM	Duncan's group number*	N	Treatment***	Mean uterine wet weight (mg) ±SEM
1	33	control	23.7±0.8	14	8	NO_2 8 mg/rat	31.1±2.6
2	10	estradiol	37.4±1.5	15	9	DDA 8 mg/rat	28.5±1.6
3	10	(±) 25	29.5±1.8	16	8	OH 25 mg/kg	35.5±3.0
4	10	(±) 50	31.6±2.2	17	6	SO_3Na 25 mg/kg	22.7±0.9
5	10	(-) 50	34.5±2.3	18	8	(±) NH_2 25 mg/kg	47.1±1.5
6	10	(+) 50	30.8±1.6	19	6	(-) NH_2 1 mg/kg	38.8±2.7
7	10	(±)100	37.2±1.7	20	6	(+) NH_2 1 mg/kg	24.6±2.9
8	10	(-)100	42.9±3.3	21	6	E sc .1 mg/kg	47.3±2.0
9	10	(+)100	28.7±1.2	22	5	(±)100 mg/kg 12 hr	33.6±1.6
10	10	(±)150	39.2±2.1	23	5	16 hr	33.8±2.0
11	10	(±)225	37.1±1.3	24	5	20 hr	34.2±2.3
12	8	(-)225	48.4±1.9	25	5	24 hr	36.0±1.9
13	8	(+)225	26.4±0.8				

Duncan's test result*

Group	17	1	20	13	15	9	3	6	14	4	22	23	24	5	16	25	11	7	2	19	10	8	18	21	12	p≤
																										.01
																										.05

*Statistical treatment consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Groups with like means are underlined.

**Listed as in Table 2.

***Listed as in Table 3.

Table 16. Mean uterine glycogen concentration (glycogen/uterine wet weight) for the 25 groups of immature female rats.

Duncan's group* number	N	Treatment**	Mean uterine glycogen conc. (µg/mg) ±SEM	Duncan's group* number	N	Treatment***	Mean uterine glycogen conc. (µg/mg) ±SEM
1	33	control	0.97±0.04	14	8	NO_2 8 mg/rat	1.64±0.19
2	10	estradiol	2.03±0.09	15	9	DDA 8 mg/rat	1.59±0.15
3	10	(±) 26	1.46±0.07	16	8	OH 25 mg/kg	1.90±0.13
4	10	(±) 50	1.71±0.07	17	6	SO_3Na 25 mg/kg	1.06±0.11
5	10	(-) 50	2.06±0.14	18	8	(±) NH_2 25 mg/kg	2.24±0.08
6	10	(+) 50	1.20±0.05	19	6	(-) NH_2 1 mg/kg	1.66±0.24
7	10	(±)100	2.07±0.15	20	6	(+) NH_2 1 mg/kg	1.08±0.08
8	10	(-)100	2.23±0.08	21	6	E sc .1 mg/kg	2.28±0.21
9	10	(+)100	1.49±0.07	22	5	(±)100 mg/kg 12 hr	1.75±0.10
10	10	(±)150	2.17±0.09	23	5	16 hr	2.30±0.17
11	10	(±)225	2.19±0.10	24	5	20 hr	1.85±0.21
12	8	(-)225	2.18±0.08	25	5	24 hr	1.88±0.15
13	8	(+)225	1.36±0.05				

Duncan's test result*

Group	1	17	20	6	13	3	9	15	14	19	4	22	24	25	16	2	5	7	10	12	11	8	18	21	23	p≤
																										.01
																										.05

*Statistical treatment consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Groups with like means are underlined.

**Listed as in Table 2.

***Listed as in Table 3.

Figure 9. The effect of several *o,p'*-DDT derivatives on uterine glycogen levels (µg/uterus) of immature female rats 18 hr after a single ip injection. Each point represents a mean ± its 95% confidence limits with N as shown.

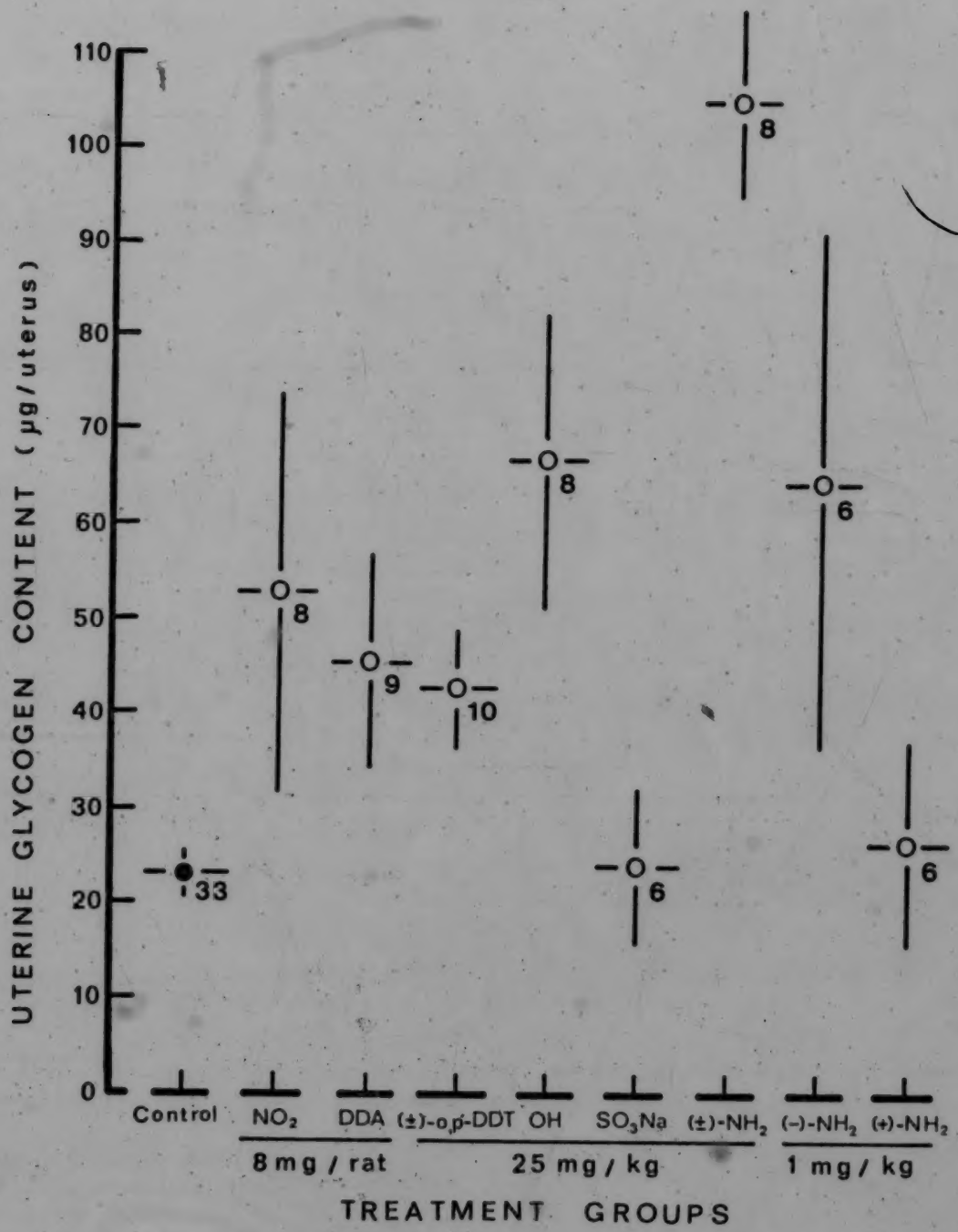

UTERINE GLYCOGEN CONTENT (µg/uterus)
110
100
90
80
70
60
50
40
30
20
10
0
33
8
9
10
8
6
8
6
Control
NO2
DDA
(±)-o,p'-DDT
OH
SO3Na
(±)-NH2
(-)-NH2
(+)-NH2
8 mg / rat
25 mg / kg
1 mg / kg
TREATMENT GROUPS

Figure 10. The effect of several *o,p'*-DDT derivatives on uterine wet weights (mg) of immature female rats 18 hr after a single ip injection. Each point represents a mean ± its 95% confidence limits with N as shown.

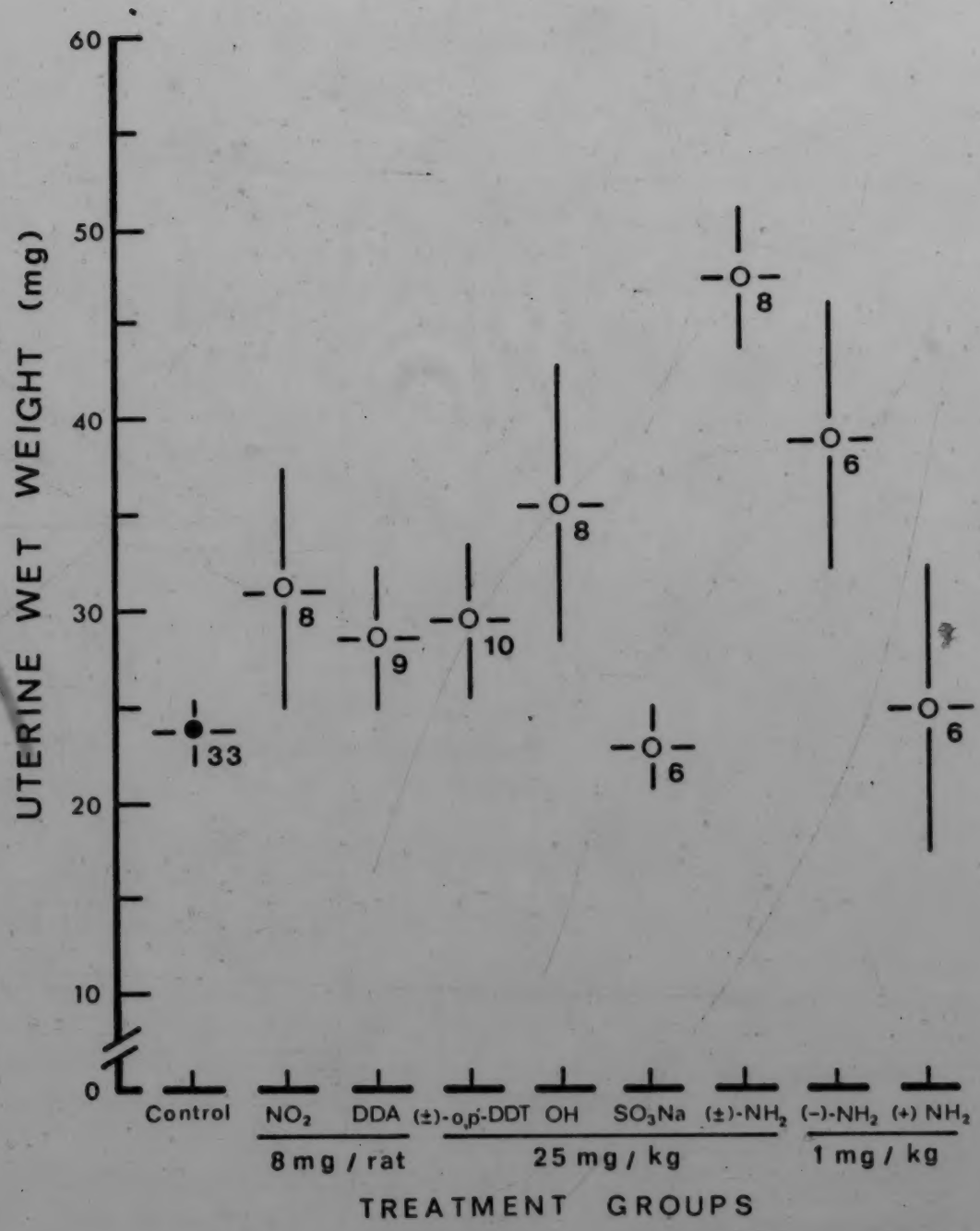
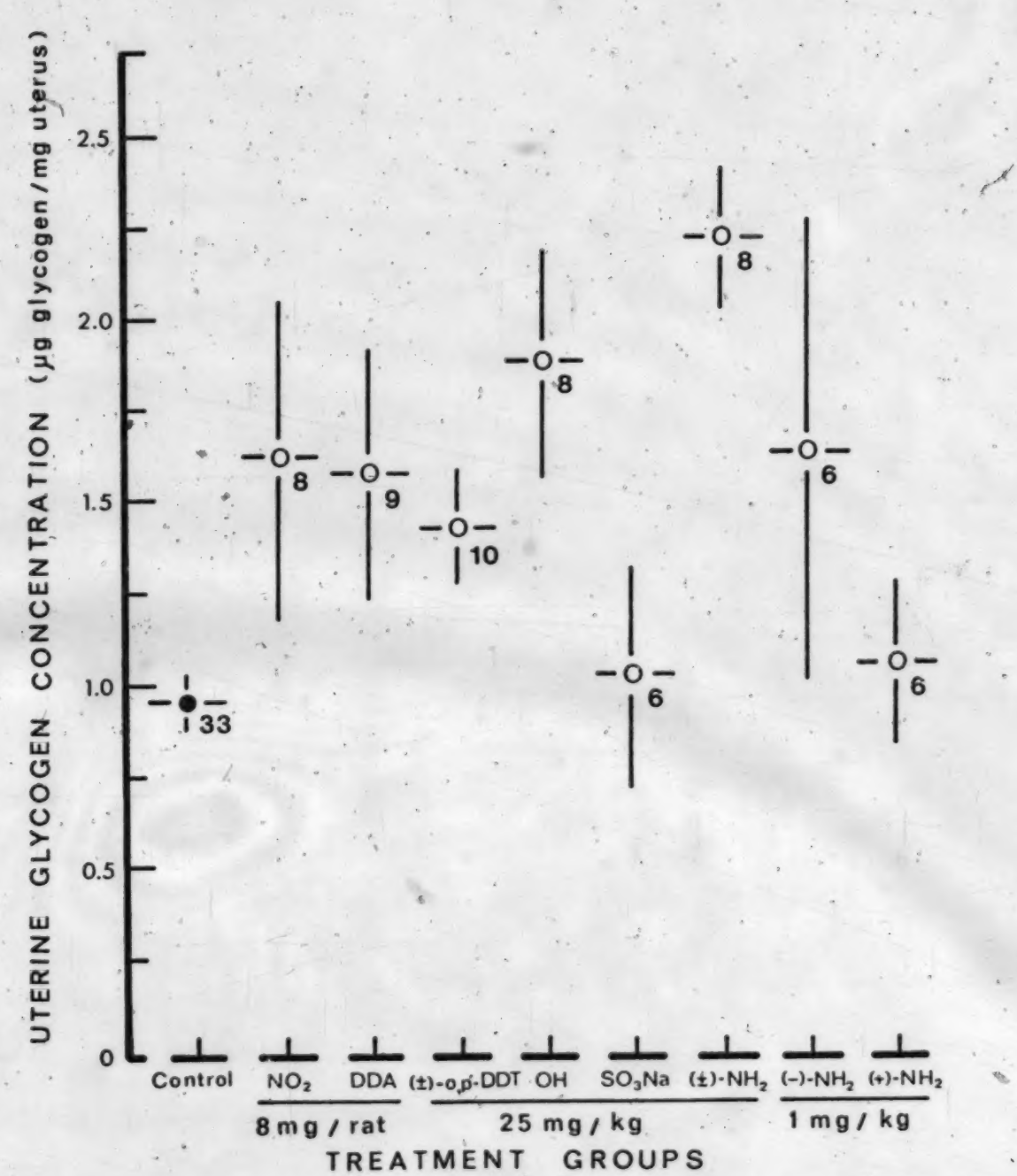

UTERINE WET WEIGHT (mg)
60
50
40
30
20
10
0
33
8
9
10
8
6
8
6
6
Control
NO2
DDA
(±)-o,p'-DDT
OH
SO3Na
(±)-NH2
(-)-NH2
(+) NH2
8 mg / rat
25 mg / kg
1 mg / kg
TREATMENT GROUPS

Figure 11. The effect of several *o,p'*-DDT derivatives on uterine glycogen concentration (µg glycogen/mg uterus) of immature female rats 18 hr after a single ip injection. Each point represents a mean ± its 95% confidence limits with N as shown.


UTERINE GLYCOGEN CONCENTRATION (µg glycogen/mg uterus)
2.5
2.0
1.5
1.0
0.5
0
33
8
9
10
8
6
8
6
6
Control
NO2
DDA
(±)-o,p'-DDT
OH
SO3Na
(±)-NH2
(-)-NH2
(+)-NH2
8 mg / rat
25 mg / kg
1 mg / kg
TREATMENT GROUPS

o,p'-DDT derivatives. The (±)-NO_2-*o,p'*-DDT, (±)-*o,p'*-DDA and (±)-SO_3Na-*o,p'*-DDT derivatives were less active estrogens than the parent (±)-*o,p'*-DDT compound. The (±)-OH-*o,p'*-DDT plus OH-*o,p'*-DDE mixture was a more active estrogen than the (±)-*o,p'*-DDT and the (±)-NH_2-*o,p'*-DDT much more active.

The (-)-NH_2-*o,p'*-DDT was an active estrogen at 1 mg/kg while the (+)-NH_2-*o,p'*-DDT apparently was not. The (+)-17β-estradiol was more active when administered via the sc route than when given by the ip route.

The (±)-*o,p'*-DDT at 100 mg/kg induced changes in the uterine parameters at 12, 16, 18, 20, and 24 hours. For the sample sizes used these changes were not significantly different from each other but the most sensitive parameter, uterine glycogen content, appeared to be maximal at 16 and 18 hours.

Female Japanese quail

ip injection-body weights

The mean initial body weights (body weights immediately prior to first injection) for the female quail are presented in Table 17. There was no significant difference among the groups for this parameter. Also in Table 17 the mean body weight changes for the three days of the study are presented. At $p \leq .05$ the (-)-*o,p'*-DDT-treated birds' mean weight loss was significantly different than the mean weight gain of the control birds.

Table 17. Mean initial body weights and mean body weight changes for the immature female Japanese quail used in the comparison of the estrogenic effects of the enantiomers of *o,p'*-DDT.

	Treatment groups*					
	(-)	(+)	E	C	(±)	
N	13	12	10	10	13	
	Mean initial body weights (g) ±SEM					
	81.5	79.6	77.2	80.2	79.4	
	±1.6	±1.7	±1.1	±2.0	±1.8	$p \leq$
						0.5
	Mean body weight changes (g) ±SEM					
	-0.2	4.7	6.6	6.9	8.2	
	±2.9	±2.8	±2.1	±0.7	±1.0	
						0.1
						0.5

*C = control and E = (+)-17β-estradiol. Bracketed signs indicate enantiomeric or racemic *o,p'*-DDT given by three daily ip injections of 5 mg.

**Statistical treatment consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Like means are underlined.

Oviducal wet weights and oviducal glycogen levels

The oviducal wet weights (Figure 12) and oviducal wet weights as proportions of body weights are presented in Table 18. As for the rats above, the enantiomers of *o,p'*-DDT differed in their estrogenic effects. The elicited increased oviducal wet weights and increased oviducal glycogen levels induced by the enantiomeric forms of *o,p'*-DDT differed significantly at $p \leq .01$ (Tables 18 and 19) with the levo form being the more active.

In contrast to the results for the rat uteri, the glycogen concentration in the quail oviducts (μg glycogen/mg oviducal wet weight) was decreased by an estrogenic response (Table 19). At $p \leq .05$ the mean oviducal glycogen concentration for the (-)-*o,p'*-DDT-treated birds was significantly lower than that of the control or (+)-*o,p'*-DDT-treated birds. That is, the (-)-*o,p'*-DDT-induced oviducal glycogen increases did not parallel the concomitant oviducal wet weight increases of Table 18 resulting in a lower concentration of the glycogen in the enlarged oviducts. The majority of the wet weight increase probably was the result of water uptake.

Serum calcium levels

Presented in Table 20 are the mean serum calcium levels from a sample of birds for three of the treatment groups. Active estrogens are known to be capable of elevating serum calcium levels in both male and female birds (Sturkie, 1965) but neither (+)-17β-estradiol (0.05 mg/day for 3 days) nor (-)-*o,p'*-DDT (5 mg/day for 3 days) affected the serum calcium levels in this study.

Figure 12. Effect of the enantiomers of *o,p'*-DDT on oviducal wet weights of immature female Japanese quail 72 hr after the first of 3 daily ip injections. Each point represents the mean ± its 95% confidence limits with N as shown.

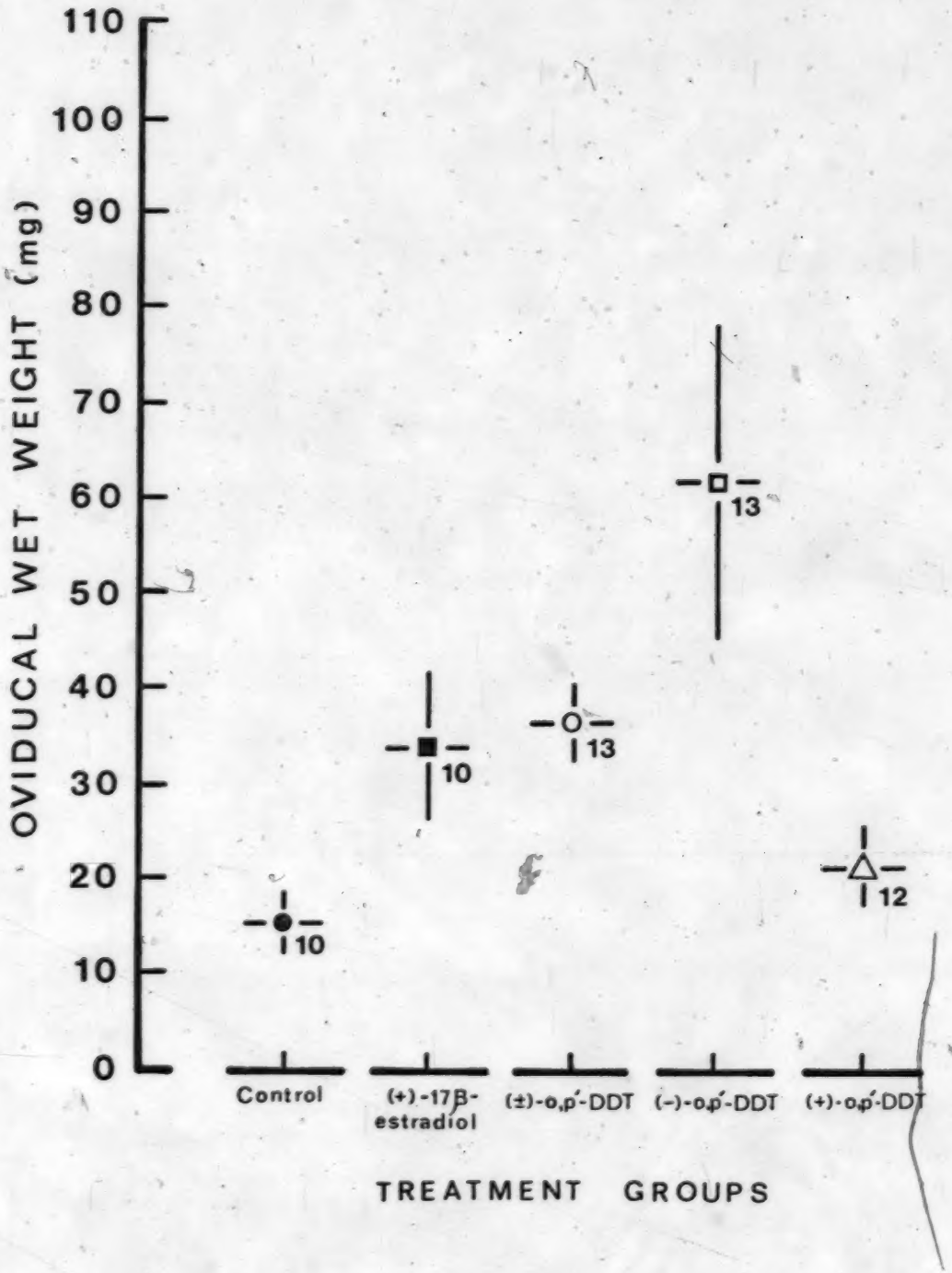

OVIDUCAL WET WEIGHT (mg)
110
100
90
80
70
60
50
40
30
20
10
0
10
10
13
13
12
Control
(+)-17β-estradiol
(±)-o,p'-DDT
(-)-o,p'-DDT
(+)-o,p'-DDT
TREATMENT GROUPS

Table 18. Mean oviducal wet weights and mean oviducal wet weights as proportions of body weights for the female Japanese quail used in the comparison of the estrogenic effects of the enantiomers of *o,p'*-DDT.

	Mean oviducal wet weights (mg) ±SEM				
	Treatment groups*				
	C	(+)	E	(±)	(-)
N	10	12	10	13	13
	15.5 ±1.4	21.6 ±1.8	33.9 ±3.3	36.6 ±1.8	61.7 ±7.5
	Mean oviducal wet weights (mg)/body weights (g) x 100 ±SEM				
	17.9 ±1.7	26.4 ±3.1	40.7 ±4.6	41.6 ±1.7	80.9 ±12.2

$p \leq$** .01 .05

*C = control and E = (+)-17β-estradiol. Bracketed sign indicates enantiomeric or racemic *o,p'*-DDT given by 3 daily ip injections of 5 mg.

**Statistical treatment consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Like means are underlined.

Table 19. Mean oviducal glycogen levels for the female Japanese quail used in the comparison of the estrogenic effects of the enantiomers of *o,p'*-DDT.

Mean oviducal glycogen levels (µg/oviduct) ±SEM					
	Treatment groups*				
	C	(+)	(±)	E	(-)
N	8	10	11	8	11
	12.3 ±2.1	14.0 ±1.8	17.4 ±1.9	19.4 ±1.6	25.1 ±3.8

$p \leq$**

.01

.05

Mean oviducal glycogen concentration (µg)/oviducal wet weights (mg) ±SEM				
0.73 ±.09	0.69 ±.14	0.47 ±.05	0.62 ±.08	0.44 ±.02

.01

.05

*C = control and E = (+)-17β-estradiol. Bracketed sign indicates enantiomeric or racemic *o,p'*-DDT given by 3 daily ip injections of 5 mg.

**Statistical treatment consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Like means are underlined.

Table 20. Mean serum calcium levels (ppm) in three treatment groups of the immature female Japanese quail.

Mean serum calcium (ppm) ±SEM				
	Treatment groups*			
	E	C	(-)	
N	5	6	6	
	48.0	50.2	55.8	
	±4.8	±7.3	±3.5	p≤**
				.05

*C = control and E = (+)-17β-estradiol. Bracketed sign indicates enantiomer of *o,p'*-DDT given by 3 daily ip injections of 5 mg.

**Statistical treatment consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Like means are underlined.

Liver, ovary and adrenal weights

Three other organs were excised from a sample of the female quail to see if the *o,p'*-DDT had affected their wet weights. The data of Table 21 indicate that the treatments used were without effect on the wet weight of the livers, ovaries or adrenals ($p \leq .01$).

Oral route-feed

The results of the study in which (±)-*o,p'*-DDT at 200 ppm in the feed was administered to immature female quail are presented in Table 22. The data were analysed using the Student's t-test to compare the values obtained for the control versus DDT-treated birds for each parameter. In both the 3 day and 7 day treatments the 200 ppm (±)-*o,p'*-DDT was ineffective in altering any of the measured parameters. That is, for this administration of (±)-*o,p'*-DDT no estrogenic activity was evident as measured by oviducal wet weights, oviducal lengths or serum calcium levels. The serum calcium levels found were higher than those reported in Table 20 for female birds or than those below for the male birds.

Oral route-capsules

The effects of capsules of (-)- or (+)-*o,p'*-DDT administered to immature female quail are shown in Table 23. The (-)-*o,p'*-DDT was estrogenic in inducing increases in oviducal wet weights and oviducal lengths while the (+)-*o,p'*-DDT was apparently inactive. Similar residues of both enantiomers were found in the uropygial glands but more of the levo enantiomer seemed to have concentrated in the fat samples. The small sample size prohibited a statistical analysis of

Table 21. Mean liver weights, mean liver weights/body weights, mean ovary weights, mean ovary weights/body weights, mean adrenal weights and mean adrenal weights/body weights for immature female Japanese quail given ip injections of (+)-17β-estradiol or *o,p'*-DDT.

	Treatment groups*					
	C	E	(±)	(-)	(+)	
N	7	7	8	8	8	
	Mean liver wet weights (g) ±SEM					
	2.57	2.77	2.97	2.84	2.77	
	±.07	±.15	±.20	±.23	±.13	$p \leq$**
						.05
	Mean liver wet weights (g)/body weights (g) x 100 ±SEM					
	2.94	3.18	3.38	3.23	3.21	
	±.10	±.10	±.20	±.15	±.09	
						.05
	Mean ovary wet weights (mg) ±SEM					
N	8	7	13	12	11	
	21.6	19.7	21.9	20.6	22.9	
	±2.0	±2.3	±1.9	±1.8	±1.8	
						.05
	Mean ovary wet weights (mg)/body weights (g) x 100 ±SEM					
	24.2	23.0	24.9	25.7	26.6	
	±2.1	±2.8	±1.9	±2.4	±2.1	
						.05

Table 21. Continued

	C	E	(±)	(-)	(+)	
			Mean adrenal wet weights (mg) ±SEM			
N	7	6	6	7	6	
	5.4	6.4	6.4	6.5	7.5	
	±.5	±.5	±.5	±.7	±1.1	$p \leq$
						.05
	Mean adrenal wet weights (mg)/body weights (g) x 100 ±SEM					
	6.2	7.4	7.4	7.8	9.0	
	±.6	±.6	±.6	±1.0	±1.3	
						.01
						.05

*C = control and E = (+)-17β-estradiol. Bracketed sign indicates enantiomeric or racemic *o,p'*-DDT given in three daily ip injections of 5 mg.

**Statistical treatment consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Like means are underlined.

Table 22. Effect of 200 ppm of dietary (±)-*o,p'*-DDT on several parameters of immature female Japanese quail.

Treatment groups			
3 day control	3 day (±)-*o,p'*-DDT	7 day control	7 day (±)-*o,p'*-DDT
N 6	6	7	7
Mean initial body weights (g) ±SEM			
54.1 ± 2.7	55.3 ± 1.5	56.8 ± 3.4	58.3 ± 2.4
t* = 0.3724		t = 0.3632	
Mean body weight gains (g) ±SEM			
10.2 ± 0.3	10.3 ± 1.0	23.7 ± 2.9	25.1 ± 1.2
t = 0.0930		t = 0.4510	
Mean oviducal wet weights (mg) ±SEM			
10.9 ± 0.6	11.6 ± 0.5	12.5 ± 1.1	14.9 ± 1.5
t = 0.9530		t = 1.4742	
Mean oviducal lengths (cm) ±SEM			
3.8 ± 0.1	3.9 ± 0.1	4.2 ± 0.1	4.2 ± 0.2
t = 0.4861		t = 0.3820	
Mean liver wet weights (g) ±SEM			
2.80 ± 0.22	2.65 ± 0.18	3.46 ± 0.18	3.41 ± 0.22
t = 0.5275		t = 0.2181	
Mean serum calcium levels (ppm) SEM			
89.0 ± 1.3	91.0 ± 3.0	95.7 ± 2.3	92.3 ± 1.7
t = 0.6049		t = 1.1931	

*t-value for Student's t-test.

Table 23. Effect of orally administered capsules of (-)- or (+)-*o,p'*-DDT on several parameters in immature female Japanese quail.

Compound	Initial body weight (g)	Body weight gain (g)	Oviducal weight (mg)	Oviducal length (cm)	Serum calcium (ppm)	*o,p'*-DDT residues (ppm)*	
						fat	uropygial gland
	66.2	9.9	16.8	4.0	87	136.2	185.8
(+)-*o,p'*-DDT	65.3	11.4	16.7	4.4	85	194.9	150.6
	76.7	13.9	21.3	3.7	87	394.2	245.5
Mean	69.4	11.7	18.3	4.0	86.3	241.8	194.0
	73.3	11.2	57.7	5.1	91	405.7	137.7
(-)-*o,p'*-DDT	68.9	15.0	40.0	4.9	83	385.3	111.1
	72.8	11.6	115.6	6.0	155	726.0	302.6
Mean	71.7	12.6	71.1	5.3	109.7	505.7	183.8

*Lipid weight basis (Appendix 6).

the residue data but certainly both enantiomers were transported within the birds' bodies. Interestingly, the (-)-*o,p'*-DDT-treated bird with the highest residues of *o,p'*-DDT in the fat and uropygial gland had a serum calcium level higher than the other 5 birds of this study and the other 26 birds of the treated feed study (Table 22). This bird as well had exhibited the greatest estrogenic response to the *o,p'*-DDT observed in this study.

Male Japanese Quail

sc injection - testicular wet weights

The effect of sc injections of 4 doses of (+)-17β-estradiol on the testicular wet weights of male quail switched from a short (8L:16D) to a long (14L:10D) photoperiod is shown in Figure 13 and Table 24. Only the 0.5 mg/2 days dose of the estradiol significantly ($p \leq .01$) prevented the expected increase of testes wet weights. The effect of the 0.05 mg/2 days dose of the estradiol approached significance while the enantiomers of *o,p'*-DDT were without effect on the testes weights.

ip injection - body weights

The mean initial injection body weights for the second group of male birds are presented in Table 25. There was no significant difference among groups for this parameter. The mean weight changes for the various treatment groups for the 10 day study period are presented in the same table. The two groups treated with the two doses of (+)-17β-estradiol differed ($p \leq .01$) from each other but neither mean differed from the control at $p \leq .01$. At $p \leq .05$ the group treated with

Figure 13. The effect of sc injections of (+)-17β-estradiol or the enantiomers of *o,p'*-DDT on light-stimulated testicular wet weight increases in Japanese quail switched from a short to a long photoperiod. The testes weights expressed as proportions of body weights are plotted for each bird and means for the estradiol-treated birds are joined.

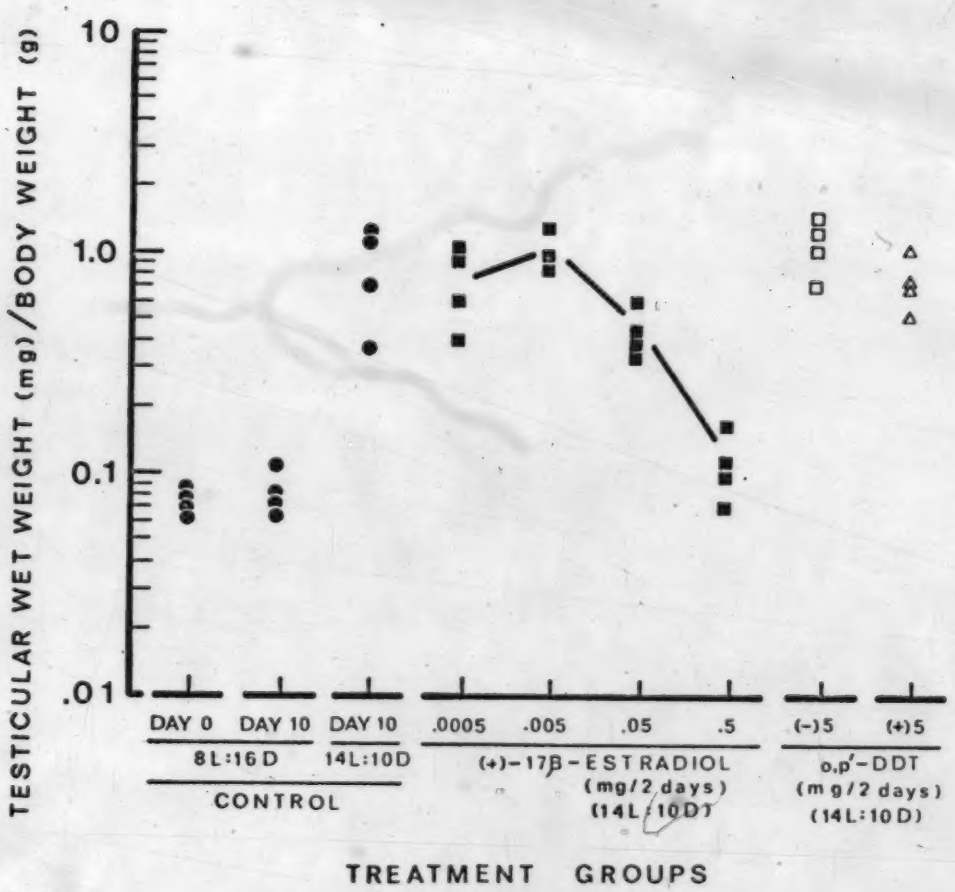

TESTICULAR WET WEIGHT (mg)/BODY WEIGHT (g)
10
1.0
0.1
.01
DAY 0
DAY 10
DAY 10
.0005
.005
.05
.5
(-)5
(+)5
8L:16D
14L:10D
CONTROL
(+)-17β-ESTRADIOL
(mg/2 days)
(14L:10D)
o,p'-DDT
(mg/2 days)
(14L:10D)
TREATMENT GROUPS

Table 24. Mean testes wet weights and mean testes wet weights as proportions of body weights for Japanese quail switched from a short to a long photoperiod and given sc injections of (+)-17β-estradiol or the enantiomers of *o,p'*-DDT.

	Mean testes weights (mg) ±SEM Treatment groups*									
	Control 8L:16D day 0	Control 8L:16D day 10	E (.5 mg) 14L:10D day 10	E (.05 mg) 14L:10D day 10	(+) 5 mg 14L:10D day 10	E (.0005 mg) 14L:10D day 10	Control 14L:10D day 10	E (.005 mg) 14L:10D day 10	(-) 5 mg 14L:10D day 10	
N	4	4	4	3	4	4	4	3	4	
	7.5	10.5	14.5	56.5	91.8	92.0	107.6	131.8	133.0	
	±.7	±1.2	±2.3	±10.7	±14.0	±16.9	±25.9	±10.2	±17.7	
										p≤**
										.01
										.05
	Mean testes weights (mg)/body weights (g) ±SEM									
	0.08	0.09	0.12	0.48	0.81	0.79	0.91	1.09	1.16	
	±.00	±.01	±.02	±.07	±.11	±.15	±.20	±.14	±.15	
										.01
										.05

*E = (+)-17β-estradiol. Bracketed sign indicates enantiomer of *o,p'*-DDT. The doses/2 days, photoperiods and duration of treatment are shown.

**Statistical treatment consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Like means are underlined.

Table 25. Mean initial injection body weights and mean body weight changes in male Japanese quail given ip injections of (+)-17β-estradiol or *o,p'*-DDT.

	Mean initial injection body weights (g) ±SEM Treatment groups*									
	E (.05 mg) 14L:10D day 10	Control 8L:16D day 10	Control 14L:10D day 10	(±) 5 mg 14L:10D day 10	(+) 5 mg 14L:10D day 10	(±) 10 mg 14L:10D day 10	(-) 5 mg 14L:10D day 10	E (.5 mg) 14L:10D day 10	Control 8L:16D day 0	
N	6	10	9	7	7	6	8	8	7	
	106.2 ±4.6	110.9 ±2.6	112.3 ±3.3	107.1 ±3.5	105.3 ±3.1	107.3 ±1.4	110.9 ±2.3	106.0 ±2.7	112.5 ±4.7	$p \leq$**
										.05
	Mean body weight changes (g) ±SEM									
	-6.9 ±3.7	-4.6 ±1.5	-4.1 ±1.8	-2.1 ±1.1	-1.1 ±1.1	-0.8 ±1.3	-0.2 ±1.9	2.6 ±1.9	---	.01
										.05

*E = (+)-17β-estradiol. Bracketed sign indicates enantiomeric or racemic *o,p'*-DDT. The doses/2 days, photoperiods and duration of treatment are shown.

**Statistical treatment consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Like means are underlined.

0.5 mg/2 days of (+)-17β-estradiol gained significantly more weight than the controls.

Testicular wet weights

The effect of two dose levels of (+)-17β-estradiol, two dose levels of (±)-*o,p'*-DDT and one dose level of (-)-*o,p'*-DDT and (+)-*o,p'*-DDT on the testicular wet weights of the ip injected male quail is shown in Table 26 and Figure 14. At $p \leq .01$ none of the compounds tested affected the testicular wet weights but at $p \leq .05$ the 0.5 mg/2 days of the estradiol significantly offset the expected weight increase. Thus the estrogenic activity of *o,p'*-DDT was not evident in the male birds for this parameter.

Serum calcium levels

Selected serum samples originating from birds of both the sc and ip injection studies were analyzed for calcium levels. Only the highest dose of (+)-17β-estradiol (0.5 mg/2 days) was able to induce ($p \leq .01$) an increase in the serum calcium (Table 27). The *o,p'*-DDT treatments were without effect.

Testis histology

Histological examination of testes from the various treatment groups (Table 28) revealed no gross abnormalities in the morphology of seminiferous tubules regardless of the treatment. The maturity of the testes was classified as shown in Table 28 and only the group treated with (+)-17β-estradiol (0.5 mg/2 days) exhibited a delay in maturation when compared to the control.

Table 26. Mean testes wet weights and mean testes wet weights as proportions of body weights for Japanese quail switched from a short to a long photoperiod and given ip injections of (+)-17β-estradiol or *o,p'*-DDT.

	Mean testes wet weights (mg) ±SEM Treatment groups*									
	Control 8L:16D day 0	Control 8L:16D day 10	E (.5 mg) 14L:10D day 10	(±) 5 mg 14L:10D day 10	(±) 10 mg 14L:10D day 10	(-) 5 mg 14L:10D day 10	E (.05 mg) 14L:10D day 10	Control 14L:10D day 10	(+) 5 mg 14L:10D day 10	
N	11	8	8	7	6	8	6	9	7	
	14.8 ±2.4	18.0 ±6.2	194.2 ±53.7	282.2 ±56.7	318.0 ±58.9	330.1 ±51.3	390.2 ±168.5	441.2 ±65.2	443.2 ±37.0	$P \leq$**
										.01
										.05
	Mean testes weights (mg)/body weights (g) ±SEM									
	0.14 ±.02	0.16 ±.05	1.86 ±.55	2.66 ±.50	2.98 ±.54	2.96 ±.42	3.77 ±1.64	4.05 ±.56	4.29 ±.40	
										.01
										.05

*E = (+)-17β-estradiol. Bracketed sign indicates enantiomeric or racemic *o,p'*-DDT. The doses/2 days, photoperiods and duration of treatment are shown.

**Statistical treatment consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Like means are underlined.

Figure 14. The effect of ip injections of (+)-17β-estradiol or *o,p'*-DDT on light-stimulated testicular wet weight increases in Japanese quail switched from a short to a long photoperiod. The mean testes weights/body weights are plotted ± their 95% confidence limits.

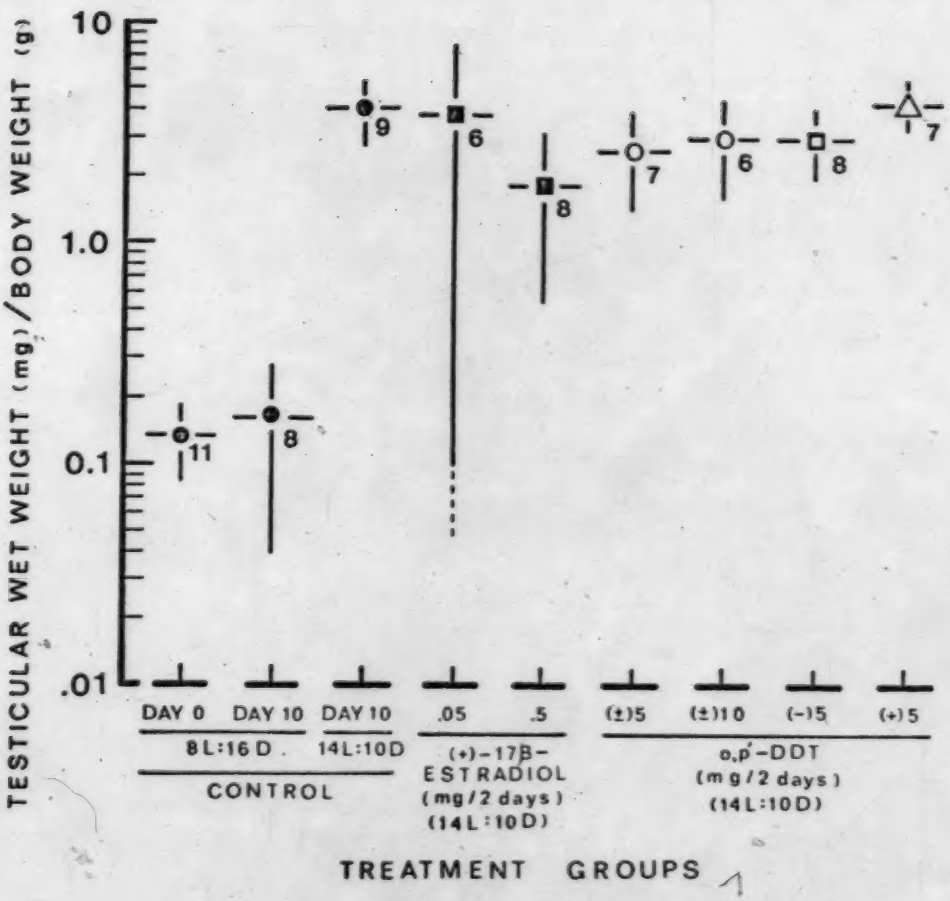

TESTICULAR WET WEIGHT (mg)/BODY WEIGHT (g)
10
1.0
0.1
.01
11
8
9
6
8
7
6
8
7
DAY 0
DAY 10
DAY 10
.05
.5
(±)5
(±)10
(-)5
(+)5
8L:16D
14L:10D
CONTROL
(+)-17β-ESTRADIOL (mg/2 days) (14L:10D)
o,p'-DDT (mg/2 days) (14L:10D)
TREATMENT GROUPS

Table 27. Mean serum calcium levels in male quail switched from a short to a long photoperiod and treated with (+)-17β-estradiol or *o,p'*-DDT by the sc or ip injection route.

	Mean serum calcium levels (ppm) ±SEM								
	(+) 5 mg 14L:10D day 10	(-) 5 mg 14L:10D day 10	Control 8L:16D day 0	(+) 5 mg 14L:10D day 10	E (.05 mg) 14L:10D day 10	Control 14L:10D day 10	Control 8L:16D day 10	E (.5 mg) 14L:10D day 10	
N	4	7	5	5	6	6	6	6	
	45.5	59.6	63.0	65.6	66.2	71.0	75.7	215.8	
	±4.1	±5.1	±2.4	±9.5	±8.3	±5.1	±6.9	±24.7	p≤ .01, .05

*E = (+)-17β-estradiol. Bracketed sign indicates enantiomeric or racemic *o,p'*-DDT. The doses/2 days, photoperiods and duration of treatment are shown.

**Statistical analysis consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Like means are underlined.

Table 28. Histologically determined testicular maturation stages for Japanese quail switched from a short to a long photoperiod and given ip injections of (+)-17β-estradiol or an enantiomer of *o,p'*-DDT.

Treatment	Testicular wet weight (mg)	Maturation stage*
Control	13.1	II
8L:16D	28.1	II
Control	237.6	III
14L:10D	272.6	III
	276.2	III
	347.4	III
	408.6	IV
	629.2	IV
	829.2	IV
(+)-17β-estradiol	23.1	II
14L:10D	26.0	II
	105.5	II
(0.5 mg/2 days)	190.0	III
	202.1	III
	392.5	IV
(-)-*o,p'*-DDT	214.1	IV
14L:10D	221.5	IV
	226.0	III
(5 mg/2 days)	278.3	IV
	287.0	III
	457.2	IV
	631.4	IV
(+)-*o,p'*-DDT	317.0	IV
14L:10D	368.6	IV
	374.4	IV
(5 mg/2 days)	434.2	III
	476.3	IV
	562.9	IV
(±)-*o,p'*-DDT	594.6	IV
14L:10D		
(10 mg/2 days)		

*Reference: Mather and Wilson (1964).

Liver weights

Liver weights and liver weights as proportions of body weights for the male quail are presented in Table 29. At $p \leq .01$ the mean liver weight for the (-)-*o,p'*-DDT treated birds was significantly greater than the 10 day control value but not from the day 0 control value. When expressed as a proportion of body weight this effect for (-)-*o,p'*-DDT was no longer significant ($p \leq .01$) but the mean liver weight/body weight for the birds treated with (+)-17β-estradiol (0.05 mg/2 days) was significantly heavier than the 10 day control values. For the time course of this study and the sample sizes used, the *o,p'*-DDT did not alter the liver weights significantly. The fact that livers of 10 day control groups and the (+)-*o,p'*-DDT-treated group tended to be lighter than the day 0 control group and estrogen-treated ((-)-*o,p'*-DDT and (+)-17β-estradiol) groups may be suggestive of some effect.

Adrenal and spleen weights

Two other organs from the male quail, the adrenals and spleens, were excised to determine if the treatments of (+)-17β-estradiol or *o,p'*-DDT could elicit gross changes in the weights of these organs. The results presented in Table 30 indicate that weights of adrenals and spleens were not affected significantly ($p \leq .01$) by the treatments utilized.

Table 29. Mean liver weights and mean liver weights/body weights for male Japanese quail switched from a short to a long photoperiod and given ip injections of (+)-17β-estradiol or *o,p'*-DDT.

	Mean liver weights (g) ±SEM									
	(+) 5 mg 14L:10D day 10	Control 8L:16D day 10	Control 14L:10D day 10	(±) 5 mg 14L:10D day 10	(±) 10 mg 14L:10D day 10	E (.5 mg) 14L:10D day 10	Control 8L:16D day 0	E (.05 mg) 14L:10D day 10	(-) 5 mg 14L:10D day 10	
N	7	10	9	7	6	8	7	6	8	
	1.89	2.07	2.08	2.14	2.32	2.38	2.47	2.47	2.47	
	±.10	±.11	±.15	±.07	±.13	±.13	±.13	±.22	±.13	
										$p \leq$**
										.01
										.05

	Mean liver weights (g)/body weights (g) x 100 ±SEM								
		Control 14L:10D day 10	Control 8L:16D day 10					(-) 5 mg 14L:10D day 10	E (.05 mg) 14L:10D day 10
	1.82	1.92	1.95	2.05	2.18	2.19	2.20	2.25	2.54
	±.10	±.12	±.10	±.06	±.11	±.09	±.07	±.14	±.29
									.01
									.05

*E = (+)-17β-estradiol. Bracketed sign indicates enantiomeric or racemic *o,p'*-DDT. The doses/2 days, photoperiods and duration of treatment are shown.

**Statistical treatment consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Like means are underlined.

Table 30. Mean adrenal weights, mean adrenal weights/body weights, mean spleen weights and mean spleen weights/body weights for male Japanese quail switched from a short to a long photoperiod and given ip injections of (+)-17β-estradiol or *o,p'*-DDT.

	Mean adrenal wet weights (mg) ±SEM Treatment groups*							
	Control 8L:16D day 10	E (.5 mg) 14L:10D day 10	(±) 5 mg 14L:10D day 10	Control 8L:16D day 0	(-) 5 mg 14L:10D day 10	Control 14L:10D day 10	(+) 5 mg 14L:10D day 10	
N	10	6	5	7	6	5	6	
	7.1 ±.4	7.5 ±.8	7.7 ±1.1	7.8 ±.9	8.0 ±.5	8.4 ±1.1	9.2 ±.5	p≤**
	Mean adrenal wet weights (mg)/body weights (g) x 100 ±SEM							
	6.7 ±.4	6.9 ±.8	7.6 ±1.2	7.0 ±.8	7.0 ±.6	8.1 ±1.0	8.9 ±.6	.01 .05
	Mean spleen wet weights (mg) ±SEM							
	30.1 ±3.1	25.5 ±2.0	28.0 ±1.9	32.2 ±5.2	26.5 ±3.3	31.5 ±4.0	22.3 ±2.7	.05
	Mean spleen wet weights (mg)/body weights (g) x 100 ±SEM							
	29.0 ±2.9	23.7 ±2.3	27.2 ±0.8	28.4 ±3.8	23.3 ±2.8	30.3 ±3.6	21.5 ±2.3	.05

*E = (+)-17β-estradiol. Bracketed sign indicates enantiomeric or racemic *o,p'*-DDT. The doses/2 days, photoperiods and duration of treatment are shown.

**Statistical treatment consisted of an analysis of variance (ANOVA) utilizing Duncan's New Multiple Range Test. Like means are underlined.

DISCUSSION

The route utilized for the resolution of the enantiomeric forms of *o,p'*-DDT was both rapid and simple. The (±)-*o,p'*-DDT was obtained readily in suitable purity from technical grade DDT via crystallization from three different solvents (Haller *et al.*, 1945). The mononitration of (±)-*o,p'*-DDT was relatively specific which allowed for the isolation of a single mononitrated derivative in pure form (Figure 4) and its reduction to the amine was apparently typical of aromatic nitro compounds (Vogel, 1967). The usual "trial and error" technique (Wilen, 1971) resulted in the discovery that optically pure (+)-10-camphorsulfonic acid would yield stable disastereomeric salts which could be crystallized from an appropriate solvent (acetonitrile).

The diastereomeric salts were separated using both a crystallization and a reflux technique but for this resolution the reflux technique had obvious advantages over the more common crystallization method with respect to both the amount of time and number of treatments required. Experimentation indicated, however, that more labile salts such as those of carboxylic acids (mandelic or tartaric acids) and the (±)-NH_2-*o,p'*-DDT would not withstand the rather harsh conditions of the reflux system although the choice of solvent may have been critical.

The deamination of the NH_2-*o,p'*-DDT enantiomers to the corresponding

o,p'-DDT enantiomers was effected in high yield by treating the diazonium salts with hypophosphorous acid (Sagar *et al.*, 1972). While it was not possible to estimate the enantiomeric purity of the resolved amines by NMR using Siever's reagent, the similarity of the magnitude of the specific rotations for both the amine (-171.0 and 166.4°) and parent compound (-17.9 and 17.7°) would suggest that the resolution may have been complete at least for the levo form. The specific rotation of the (-)-amine was not increased by additional reflux or crystallization of its (+)-10-camphorsulfonate salt.

The fact that an optical resolution had taken place was verified in the biological studies of the enantiomers of both NH_2-*o,p'*-DDT and *o,p'*-DDT. Both racemic compounds were found to be estrogenically active in immature female rats but this estrogenic activity was subject to the phenomenon of biological stereospecificity.

The estrogenic activity of racemic *o,p'*-DDT has been documented repeatedly in recent years (see Introduction) but it was not until 1975 that biological stereospecificity was reported to be of potential significance to the estrogenic effects of this compound (Kupfer, 1975; McBlain and Wolfe, 1975). It may be that the property of optical activity in *o,p'*-DDT and its analogs had been overlooked until 1975 since no previously published reports had contained any reference to this property. The resolution of the enantiomeric forms of NH_2-*o,p'*-DDT and *o,p'*-DDT has permitted the comparison of the relative estrogenic activities of these optical isomers.

The sensitivity of the immature female rat uterus to various exogenous estrogens (Astwood, 1938; Bitman and Cecil, 1970; Cecil *et al.*,

1971a) made it an excellent test system for the determination of the estrogenic activities of the *o,p'*-DDT enantiomers as well as several *o,p'*-DDT derivatives. Both uterine wet weights and uterine glycogen levels, two easily measured parameters, increased markedly only 18 hours after administration of active estrogens.

For the present study of the estrogenic effects of *o,p'*-DDT and its derivatives, immature female rats, 20-21 days old, weighing between 35 and 50 g were utilized. The mean initial body weights of the rats in the various treatment groups (Tables 9 and 10) did not differ from the controls ($p \leq .01, .05$) with the exception that the rats treated with 225 mg/kg of (+)-*o,p'*-DDT in the first part of this study (Table 9) were statistically lighter ($p \leq .05$) than the control animals. However, even if these lighter rats were somewhat less capable of responding to an estrogen it is doubtful if the differences in body weights could account for the differences in estrogenic responses to (-)-, (±)- and (+)-*o,p'*-DDT observed at 225 mg/kg (Figures 6-8 and Table 13). It would seem that the estrogenic responses of the rats to the various chemical treatments were the result of the estrogenic potencies of the compounds under the experimental conditions utilized and were not related to the maturity of the rats as reflected in body weights.

The mean 18 hour body weight changes for the 25 treatment groups of rats (Tables 11 and 12) ranged from 1.0 to 5.3 g. Several of these mean weight gains differed significantly from each other but none of them differed from the control value, 3.7 g, at $p \leq .01$. For $p \leq .05$ the rats treated with (-)- or (+)-*o,p'*-DDT at 225 mg/kg had mean weight gains significantly lower than the controls (Table 11) as did the (±)-*o,p'*-DDA and (±)-NH_2-*o,p'*-DDT-treated groups of Table 12. The adverse

behavioral reactions exhibited by rats immediately following an injection of (±)-*o,p'*-DDA or (±)-SO_3Na-*o,p'*-DDT was suggestive of a toxic effect of these compounds and the animals so treated along with those injected with the higher doses of *o,p'*-DDT had mean weight gains which tended to be lower than those of the other groups. While these findings may be regarded as evidence of a toxic action of the compounds it is difficult to evaluate the data since 18 hours was a short time over which to measure the relatively insensitive index, body weights. It is difficult to explain why some groups had mean weight gains greater than the controls (not statistically significant) but this probably was not an estrogenic effect of the administered compounds since the two estradiol-treated groups had mean weight gains almost identical to the control value.

The dose-response relationships for the effect of (±)-*o,p'*-DDT on the uterine parameters, wet weight and glycogen levels in the immature rats for the present study (Figures 6 and 7 and Table 13) were similar to those reported earlier by Cecil *et al.* (1971a). While the uterine wet weight increases induced by (±)-*o,p'*-DDT in the current study were almost identical to those reported by Cecil *et al.* (1971a), the maximal uterine glycogen levels in the present study were lower. This may be explained by the fact that both the strain of rats and route of administration of the compounds differed between the two studies.

The (±)-*o,p'*-DDT-induced increases in uterine glycogen concentration (Figure 8 and Table 13) were calculated on the basis of the uterine glycogen levels (µg/uterus) as proportions of the uterine wet weights (mg) rather than on a dry weight basis. An examination of the data of Cecil *et al.* (1971a) had revealed that uterine dry weights changed little

for doses of (±)-*o,p'*-DDT above 1 mg/rat (about 22 mg/kg for a 45 g rat) and therefore a presentation of uterine glycogen as proportions of uterine dry weights would have paralleled the simple presentation of uterine glycogen levels alone. Both sham-injected and (+)-17β-estradiol-injected animals served as "controls" for these studies of estrogenic activity. Obviously the well-known estrogenic activity of (±)-*o,p'*-DDT in immature female rats has been verified in the present study.

The estrogenic activities of both (±)-*o,p'*-DDT and (+)-17β-estradiol in immature female Japanese quail in the present study (Figure 12 and Tables 18 and 19) were almost identical to those reported previously (Bitman *et al.*, 1968). In both studies the compounds were administered by ip injection and it is likely that Cooke (1970) had failed to induce estrogenic responses in his female quail treated with (±)-*o,p'*-DDT because he administered the compound intramuscularly.

The choice of route for the administration of compounds was critical to the results obtained herein. Cecil *et al.* (1971a) administered both (+)-17β-estradiol and (±)-*o,p'*-DDT to their rats via the sc route but in preliminary experiments conducted at the outset of the current studies no response was obtained for (±)-*o,p'*-DDT via this route. All experimental conditions were thought to be the same as those of Cecil *et al.* (1971a) except that the site of their sc injections was not known. The combined factors of route and solvent were also important as sc injections of the (±)-*o,p'*-DDT in DMSO did elicit estrogenic responses. From Tables 14-16 it can be seen that the (+)-17β-estradiol in the current study was more active in rats when injected via the sc route than via the ip route. A preliminary study had indicated that this sc/ip relationship was true in immature female quail as

well and from Figures 13 and 14 and Tables 24 and 26 it can be seen that the administered estradiol was again apparently more effective by the sc rather than the ip route in male quail.

The ip route in birds was not completely satisfactory for the administration of the compounds tested. The air sacs in birds may be ruptured during the injection or may prevent an even dispersal of the injected solution within the coelom. Also it was relatively easy to pierce the liver, gall bladder or intestine during the injection. If the liver had been pierced the birds usually died of massive hemorrhage shortly following the injection. The only birds which died during this study, died as a result of an injury during an injection and data collected on them to that point were eliminated from the statistical treatment of the results. If the gall bladder was ruptured the birds lost weight but rarely died. In a few cases the birds with pierced gall bladders exhibited higher than normal estrogenic responses probably because the bile emulsified the olive oil solvent vehicle. Such birds were found in all treatment groups so did not interfere with the data analyses.

The effect of the ip injections on the birds' health can be seen in the weight changes for both the females (Table 17) and the males (Table 25). All groups of females were immature and growing and therefore exhibited mean weight gains except for the group treated with (-)-*o,p'*-DDT (5 mg/day for 3 days). The male birds had almost reached their maximal body weights and because of this and because of the extra treatments (injected every other day for 10 days) all groups had mean weight losses except for the group treated with the higher dose of estradiol. For both the male and female birds the mean weight changes

undoubtedly were affected by the injection techniques used and no assumptions about the toxicities of the injected compounds can be made.

The mean body weights for the groups of quail at the time of first injection did not differ significantly within either the female (Tables 17 and 22) or male (Table 25) studies. However, the body weights of the birds differed according to the time they were obtained from Bio-Science Animal Services. That is, the immature female quail used for the ip route study weighed between 70 and 93 g while those used in the oral route study weighed only 44-77 g. Similarly, the male birds for the sc study weighed 64-101 g while those of the ip study weighed 96-113 g. Therefore while there was internal uniformity within the quail studies comparisons of results among the studies should be made taking into consideration these weight differences. The author believes that the various hatches of quail differed in size because of differences in the manner in which they were handled by the BioScience Animal Services personnel.

The (±)-*o,p'*-DDT administered ip to the immature female quail, 5 mg/day for 3 days to a total of approximately 200 mg per kg of body weight, elicited mean oviducal wet weight increases about half those observed for (-)-*o,p'*-DDT (Table 18). Alternatively, in rats, a dose of 200 mg/kg of (±)-*o,p'*-DDT resulted in maximal increases in the uterine parameters measured. The (+)-17β-estradiol given to the immature female quail, 0.05 mg/day for 3 days, a total of approximately 20 mg per kg of body weight, induced oviducal changes similar to those for (±)-*o,p'*-DDT. In the immature female rats 0.1 mg/kg of (+)-17β-estradiol gave almost maximal uterine responses by the ip route. These findings indicate that the amounts of (±)-*o,p'*-DDT required for uterine

and oviducal responses in the rats and quail respectively are within an order of magnitude while those for the (+)-17β-estradiol differ by at least two orders of magnitude. That is, while the immature female rats and quail were equally sensitive to (±)-*o,p'*-DDT, the quail, possibly for some inherent inability to respond, were less sensitive to the effects of (+)-17β-estradiol. The glycogen levels in the rat uteri were much more sensitive to administered estrogens than the glycogen levels in the quail oviducts (Figure 6 and Tables 14 and 19).

The methodology of the current quail study included a consideration of the photoperiod since it has been established that the sexual maturation of Japanese quail can be controlled by regulating the light regime (Follett and Farner, 1966; Tanaka *et al.*, 1965; Wilson *et al.*, 1962). The female quail responded to the treatments of (±)-*o,p'*-DDT and (+)-17β-estradiol despite the fact that a nonstimulatory 8L:16D photoperiod (Tanaka *et al.*, 1965) was used.

Administration of (±)-*o,p'*-DDT by the ip route to male quail failed to offset a light-stimulated increase in testicular wet weights (Figure 14 and Table 26). That is, male quail held on a nonstimulatory 8L:16D photoperiod were switched to a stimulatory (Tanaka *et al.*, 1965) photoperiod of 14L:10D and the control birds exhibited the expected increase in testicular wet weights. While 0.5 mg of (±)-17β-estradiol given every other day for 10 days prevented the testicular growth ($p < .05$) the (±)-*o,p'*-DDT at 5 or 10 mg/2 days for 10 days was without effect.

The daily doses of (±)-*o,p'*-DDT and (-)-*o,p'*-DDT used in these male birds were similar to the doses which were estrogenically active in the females. The (+)-17β-estradiol was not as effective via the ip

route as it had appeared to be via the sc route (Figure 13 and Table 24) as discussed above. The testes weights for all groups in the sc study were lighter than those of the ip study probably because the birds were lighter as well (see above discussion). For both studies (Tables 13 and 14) large variations are obvious in the mean weights of testes from birds which had responded to the light stimulus. These variations probably resulted from individual variations among the responding birds. The responses appear to be logarithmic in nature and the birds were sacrificed before any of them had had time to attain maximal responses. Data from near the mid-points of time-response and dose-response studies should be expected to exhibit considerable variability. The histologically determined testicular maturation (Mather and Wilson, 1964) gave an accurate indication of the effect of the estradiol (Table 28) on the testes. That is, the testes were classified as class II if resting spermatogonia and only a few spermatocytes were present, class III if many spermatocytes were present and class IV if spermatids had appeared. While the 14L:10D control testes were all classified as stage III or IV the estradiol-treated birds had testes ranging from stage II to stage IV (Table 28). The undeveloped stage II testes of the estradiol-treated birds were correlated to low weights of the testes and histologically these testes were similar to those of the short day control birds examined. None of the testes examined showed evidence of histological damage as seen for the estrogenic insecticide Kepone (Eroschenko and Wilson, 1975).

Other workers (Campbell and Mason, 1975; Gellert *et al.*, 1974); Gustafsson and Stenberg, 1976; Ware, 1975) have reported that the vertebrate male system is apparently resistant to the estrogenic effects

of *o,p'*-DDT. It is important to consider, however, that while in the female system an estrogenic compound acts directly on the reproductive system, in male vertebrates estrogens must act indirectly via the hypothalamo-pituitary axis.

Estradiol and testosterone levels in female and male vertebrates respectively are controlled through feedback mechanisms via steroid receptors in the hypothalamus which thereby controls LH and FSH release by the pituitary gland. The fact that (+)-17β-estradiol prevented testicular maturation in male quail means that the estradiol must have interacted with the hypothalamic steroid receptors to prevent the pituitary release of LH and FSH. Gellert *et al.* (1972) demonstrated that (±)-*o,p'*-DDT could apparently affect LH but not FSH levels in ovariectomized rats but this is the only evidence to suggest that *o,p'*-DDT can reach and interact with hypothalamic steroid receptors. In the present study of male quail 0.05 mg of (+)-17β-estradiol every other day for 10 days (ip) did not affect the testicular development while 0.5 mg/2 days for 10 days (5 times the daily dose estrogenically active in females) was effective. Perhaps a similar 5-fold increase in the dose of (±)-*o,p'*-DDT between male and female birds would have been required to have elicited a response. Several other factors could have affected the response of the male birds to (±)-*o,p'*-DDT including its intrinsic activity, rate of transport into the brain, stereospecificity of the hypothalamic receptors and any other possible differences between the uterine and hypothalamic estrogen receptors.

None of the *o,p'*-DDT treatments affected the weights of livers or adrenal glands of the male or female birds as had been reported by Eroschenko and Wilson (1974) for the estrogenic insecticide Kepone.

Increased liver weights could have resulted in the present study from an increased synthesis of microsomal enzymes (to metabolize the *o,p'*-DDT) or from an estrogenic response. Estrogens induce fat mobilization in birds (Sturkie, 1965) and increased liver weights might be associated with this alteration in fat metabolism. Increased adrenal weights would have reflected a response to the stress of the treatments or an altered feedback between corticosterone and ACTH through an increased metabolism of the steroid by the increased liver microsomal enzyme activity. The time-courses of the experiments in the present study were probably too short to allow changes in such parameters to become evident.

In the immature female rats the levo enantiomeric forms of both NH_2-*o,p'*-DDT and *o,p'*-DDT were more active estrogens than the respective dextro forms. The induced increases in uterine wet weights and glycogen levels (Figures 6-11 and Tables 13-16) demonstrate the operation of biological stereospecificity. The (-)-*o,p'*-DDT was more active than (±)-*o,p'*-DDT while (+)-*o,p'*-DDT was less active than (±)-*o,p'*-DDT indicating that the estrogenic activity of the racemic compound resides with the levo form. Similarly, in immature female Japanese quail (-)-*o,p'*-DDT increased the oviducal wet weights, lengths and glycogen levels while the (+)-*o,p'*-DDT was apparently inactive (Figure 12 and Tables 18 and 19).

The differing estrogenic activities for these enantiomers was not unexpected because the steroid hormone receptor is a protein (O'Malley and Means, 1974) and therefore inherently dissymmetric so that hormone-receptor complexes are diastereomeric in the case of asymmetric hormones.

For example, the enantiomers of 17β-estradiol benzoate differ in their estrogenic activities (Meyerson, 1971) possibly because of differences in the chemical properties of their diastereomeric associations with the estrogen receptor sites. It is also possible that one enantiomer is excluded from the receptor site by steric restrictions. For example, while the enantiomeric forms of hexestrol do not differ in their estro-activities (Collins and Hobbs, 1970), neither form is a particularly active estrogen as the estrogenic effects of hexestrol result from the action of the meso form of this compound. The differing estrogenic activities for the enantiomeric versus meso-hexestrol indicates that the estrogen receptor site has stringent steric restrictions. It is also possible that the dextro enantiomers of NH_2-*o,p'*-DDT and *o,p'*-DDT may have had a limited access to the receptor site because of such steric restrictions.

The finding that the levo form of *o,p'*-DDT possessed the R configuration (Smith, Dr. R. A.; personal commun.) had been predicted during the course of this work on the basis of comparison of the molecular models (Framework Molecular Models, Prentice-Hall, Inc.) of (+)-17β-estradiol and the enantiomers of *o,p'*-DDT. This comparison involved the speculation that the *o*-Cl ring of *o,p'*-DDT would assume the same position at the receptor site as the aromatic ring of the estradiol. When the para position of the *o*-Cl ring of *o,p'*-DDT was aligned with the 3-OH group of (+)-17β-estradiol only the R form of *o,p'*-DDT had the proper configuration to assume a position giving a molecular shape similar to that of the estradiol. It was predicted that this form was the estrogenically active levo *o,p'*-DDT.

Bitman and Cecil (1970) have postulated that a *p*-OH metabolite of

o,p'-DDT is the active estrogen so that the above alignment of the aromatic rings for the comparison of structure seemed logical. A further assumption used in this comparison was that the *o,p'*-DDT ring orientation would be similar to that revealed in the report of the crystal structure of the racemic modification of this compound (DeLacy and Kennard, 1972) and the position of the trichloromethyl group was critical in giving molecular dimensions similar to (+)-17β-estradiol. The rigidity (lack of single bond rotations) in the estradiol molecular model was obvious and it seems likely that the restricted rotation within the *o,p'*-DDT molecule (McKinney *et al.*, 1974) is an essential feature of its estrogenic activity. Similar compounds with a less restricted rotation of the aromatic rings (*o,p'*-DDA, *o,p'*-DDD and *o,p'*-DDE) are less active estrogens (Bitman and Cecil, 1970; this work, Figures 9-11 and Tables 14-16).

It is also possible that both *o,p'*-DDT enantiomers had equal access to and association with the estrogen receptors but that the differences in the properties of the diastereomeric complexes so-formed were adequate to give differing estrogenic responses in the target tissues. The differences between the hypothesized diastereomeric complexes could affect any one of the several steps in the mode of action sequence of steroid hormones (O'Malley and Means, 1974). That is, steroid hormones act by entering the cellular cytoplasm, binding with a receptor and travelling into the nucleus bound to the receptor. Once in the nucleus the steroid-receptor complex becomes associated with the chromatin and induces biosynthesis of RNA and thereby protein. One of the proteins thought to be synthesized (renewed) is the receptor protein itself and the other proteins are cell specific depending on the type of tissue and

its function. The study in which capsules of the enantiomers of *o,p'*-DDT were administered to immature female quail (Table 23) indicates that both enantiomers were transported within the birds and both would have been carried to the tissues possessing estrogen receptor sites.

Bitman and Cecil (1970) suggested that (±)-*o,p'*-DDT acted as an estrogen following hepatic para-hydroxylation of the *o*-Cl ring. In the present study a hydroxyl group was introduced into one meta position (para to the chlorine of the *o*-Cl ring) and this hydroxy derivative was a more active estrogen in the immature female rats than the parent *o,p'*-DDT molecule (Figures 9-11 and Tables 14-16). This observation supports the suggestion by Bitman and Cecil (1970) that a hydroxylated derivative of (±)-*o,p'*-DDT would be an active estrogen. However, during the preparation of this hydroxy compound some dehydrochlorination occurred so that the product consisted of about 65% (±)-OH-*o,p'*-DDT and about 32% OH-*o,p'*-DDE. It is possible that the OH-*o,p'*-DDE was the more active estrogen. This seems unlikely since *o,p'*-DDE has only moderate estrogenic activity (Bitman and Cecil, 1970) and its lack of activity compared to (±)-*o,p'*-DDT may result from the relatively greater freedom of rotation of the aromatic rings, a property not affected by hydroxylation. Neither (±)-OH-*o,p'*-DDT nor OH-*o,p'*-DDE appeared to be as estrogenically active as (+)-17β-estradiol in immature female rats for the conditions of this study.

Some further ideas regarding the structural and chemical requirements of estrogenic compounds may be gained by examining the estrogenic activities of the various derivatives of *o,p'*-DDT. While the mixture of phenolic compounds ((±)-OH-*o,p'*-DDT plus OH-*o,p'*-DDE) was estrogenically active the sulfonated derivative ((±)-SO_3Na-*o,p'*-DDT) was inactive

(Figures 9-11 and Tables 14-16) but this sulfonic acid derivative would have had a greater steric bulk. The bulky nitro group also appeared to render the (±)-*o,p'*-DDT inactive as an estrogen (Figures 9-11 and Tables 14-16) so a preliminary assessment would indicate that there could be a size restriction for estrogenic compounds in this system. However, it is likely that several other factors affected the estrogenic activities of the compounds tested.

For example, it is possible that an electronic factor may be important in determining the estrogenic activity of compounds. It has been reported that *o,p'*-DDT may associate with aromatic amino acids within biological systems through π orbital interactions (McKinney *et al.*, 1974) and such interactions could be essential in transport and receptor site associations. Since the amino acids referred to would be constituents of inherently dissymmetric proteins such transport mechanisms within the animals could be stereospecific. Furthermore aromatic substitution with activating groups would supply electrons to the π orbitals and possibly enhance the π orbital interactions with the aromatic amino acids of both transport and receptor site proteins. Lipophilic compounds such as *o,p'*-DDT undoubtedly rely on lipoproteins for transport within the circulatory system of vertebrates.

The compounds in the present study which had ring activators added to the parent *o,p'*-DDT molecule (the OH and NH_2 derivatives) were estrogenically active while the derivatives with ring deactivating substituents (the NO_2 and SO_3Na compounds) were estrogenically inactive.

Another property to consider is the ability of the derivatives to become engaged in hydrogen bonding. Both the estrogenically active OH- and NH_2-*o,p'*-DDT derivatives may hydrogen bond to transport or receptor

sites. The fact that the (±)-*o,p'*-DDA and (+)-NH_2-*o,p'*-DDT can hydrogen bond but were estrogenically inactive means that further factors must be taken into consideration when relating structure to activity.

The interpretation of the results from the type of structure-activity studies carried out in the present project is limited. It is impossible to determine the importance of such factors as release rates of various compounds from various solvent vehicles, properties of transport mechanisms within the body, affinities of the compounds for the receptor sites and the varied and numerous parameters concerning the animals used. Ideally, various solvent vehicles and routes of administration should be utilized and the findings from such experiments should be correlated with the findings of experiments on the *in vitro* binding of the compounds with estrogen receptors. A further complication of this type of testing is the possibility that the compounds may act through active metabolites in the *in vivo* system. Such metabolic processes would interfere with the interpretation of *in vitro* binding studies and it is probable that such metabolic processes would be stereospecific as well. Current methodology will not permit an accurate determination of the binding affinities of DDT-type compounds with estrogen receptors because of technical problems involved in labelling these compounds adequately (Kupfer, 1975).

The results dealing with two additional aspects of the effects of *o,p'*-DDT on Japanese quail may be of significance with regard to the environmental implications of this study. The estrogen-sensitive parameter, serum calcium content, was unaffected by treatment with (-)-*o,p'*-DDT in both male and female birds (Tables 20 and 27). A dose of 0.05 mg/day of (+)-17β-estradiol for 3 days also failed to affect the

serum calcium levels of the immature female birds. In male birds, a dose of 0.5 mg of (+)-17β-estradiol every other day for 10 days substantially increased the serum calcium levels (Table 27) indicating that the estradiol treatment had the potential to be effective in altering this parameter in the females as well. The doses of (-)-*o,p'*-DDT and (+)-17β-estradiol used in the female birds had given estrogenic responses for the measured oviducal parameters. This demonstrated that the calcium response was a less sensitive indicator of estrogenic activity, and that the doses of *o,p'*-DDT and estradiol used may have been too low. Furthermore the birds may have been too young to respond to the treatment or the time interval for the treatment may have been too short. Estrogens act in conjunction with parathyroid hormone (PTH) to elevate serum calcium levels in birds (Sturkie, 1965) and the degree of activity of the parathyroids in the birds of the present study is unknown. Interestingly, one female bird treated with capsules of (-)-*o,p'*-DDT (Figure 23) exhibited a high estrogenic oviducal response, had relatively high tissue levels of the DDT and also had an elevated serum calcium level. Obviously, more research could be done to examine the possibility that *o,p'*-DDT might affect the calcium metabolism in avian species.

Within the current study a technical problem was encountered in the determination of the serum calcium levels. Birds from which the blood samples were removed by live cardiac puncture (Tables 20 and 27) had serum calcium levels lower than similar birds from which the blood was removed following chloroform anaesthesia and direct puncture of the ventricle (Tables 22 and 23). The reason for this difference is uncertain but the effect of rupturing the aortic arches, atria and other vessels during the live cardiac puncture is unknown. Furthermore hemolysis was

observed in most of the samples taken by this method. The resultant variability in the results from these samples prevent apparently different means from being statistically different (Table 27). However, since an estrogenic response to one of the treatments utilized should have given a two to three-fold increase in serum calcium levels as seen for the male birds given the higher dose of estradiol (Table 27) it seems unlikely that the statistical interpretation of this data is biased.

When (±)-*o,p'*-DDT was administered by a more natural route (about 200 ppm in the feed) to immature female quail neither the serum calcium nor the measured oviducal parameters were affected (Table 22) similar to the results of Cecil *et al.* (1971a) and Clement and Okey (1972) for rats. This dose was far in excess of levels likely to be encountered by birds in the wild. However, the possibility that physiologically active metabolites of DDT are present in natural feeds in areas where DDT is used as an insecticide cannot be overlooked.

The birds given the 200 ppm of dietary (±)-*o,p'*-DDT consumed an average of about 15 g of feed per day which would have given a daily intake of about 3 mg of (±)-*o,p'*-DDT. When the estrogenically active levo isomer of *o,p'*-DDT was administered orally in capsules an estrogenic response was observed but the dose for this latter study was about 20 mg/day for a total of about 60 mg. However, if it is assumed that the fat makes up about 10% of the birds' weights the 3 capsule-treated birds would only have about 3.5 mg of the 60 mg of administered (-)-*o,p'*-DDT present in body fats indicating a poor absorption of the chemical from the gut or a rapid metabolism and/or excretion.

Racemic *o,p'*-DDT is a relatively poor insecticide so unless its

enantiomers act in a strongly antagonistic fashion it is doubtful if the resolved isomers are of value as insecticides. This also implies, however, that exclusion of (±)-*o,p'*-DDT from insecticidal preparations of technical grade DDT (possibly by a single crystallization from ethanol) would enhance their insecticidal toxicity while preventing environmental contamination by an active estrogen. Interestingly, both *p,p'*-DDT and *p,p'*-DDE which are thought to have adverse effects on wild avian populations (see Introduction) are respectively weak or inactive as estrogens (Bitman and Cecil, 1970). Perhaps the lack of a simple "cause and effect" relationship between *p,p'*-DDE residues and avian eggshell thickness (Cooke, 1973) could be explained if the environmental fate of (±)-*o,p'*-DDT were known.

Other xenobiotics of environmental significance are racemates (Wilkinson, 1973) and their physiological and pharmacological effects should be evaluated taking into consideration the phenomenon of biological stereospecificity.

SUMMARY

1. The enantiomeric forms of *o,p'*-DDT have been resolved and the specific rotations revealed were -17.9 and 17.7°.
2. The levo enantiomer of *o,p'*-DDT is the more active estrogen in immature female rats and Japanese quail.
3. The findings for this study provide an illustration of the phenomenon of biological stereospecificity.
4. A light-stimulated increase in testicular wet weights in immature male Japanese quail was prevented by (+)-17β-estradiol but not by *o,p'*-DDT.
5. A dose of 200 ppm of (±)-*o,p'*-DDT in the feed of immature female Japanese quail was without effect on certain estrogen-sensitive parameters.
6. Estrogenically active (±)-*o,p'*-DDT should be removed from the technical grade DDT currently used as an insecticide.

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APPENDICES

APPENDIX 1

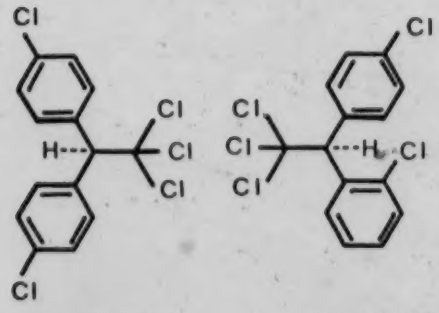
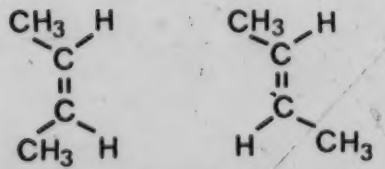
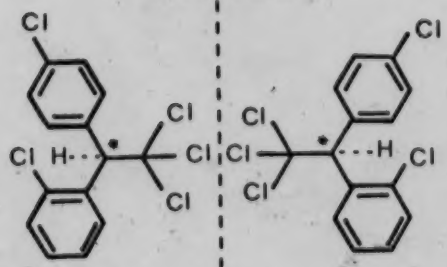
Optical isomerism

The topic of optical isomerism is discussed in most general organic chemistry texts and the following represents a brief summary from two such texts (Barker, 1971; Morrison and Boyd, 1967) as well as two more specialized texts on stereochemistry (Alworth, 1972; Eliel, 1962).

Chemical isomers are structurally different compounds having the same molecular formulae as illustrated for the case of methyl ether and ethanol in Figure 15. If this difference between isomers is caused simply by differing spatial orientations of atoms within the molecules rather than the order in which the atoms are joined, the isomers are called stereoisomers (Figure 15).

Stereoisomers are subdivided into two classes. Those that are mirror images of each other as illustrated in Figure 15 are called enantiomers while those that are not mirror images of each other are called diastereomers. Stereoisomers may also be classified according to symmetry. Molecules may be symmetric, having a plane of symmetry, axis of symmetry, center of symmetry or a rotation-reflection axis of symmetry and superimposable mirror images; or they may be dissymmetric, such that of the three types of symmetry above they may possess no element of symmetry or a simple axis of symmetry only and have

Figure 15. A simplified scheme for the classification of chemical isomers (adapted from Alworth (1972) and Morrison and Boyd (1967)). The asterisks designate asymmetric carbon atoms.

MOLECULAR FORMULA	STRUCTURAL FORMULA NAME OF COMPOUND	ISOMER TYPE
C_2H_6O	CH_3OCH_3 methyl ether; CH_3CH_2OH ethanol	STRUCTURAL ISOMERS
$C_{14}H_9Cl_5$	 p,p'-DDT; o,p'-DDT	
C_4H_8	 2-butene	DIASTEREOMERS → STEREOISOMERS
$C_{14}H_9Cl_5$	 o,p'-DDT	MIRROR → ENANTIOMERS

nonsuperimposable mirror images called enantiomers. For example, a compound containing a carbon atom bearing four different atoms or chemical groups (an asymmetric carbon atom) is dissymmetric and may exist as two enantiomeric forms with nonsuperimposable mirror images (Figure 15).

Enantiomers are optical isomers. That is, they have identical chemical properties and differ in only one physical property, the ability to rotate the plane of polarized light.[3] The rotation of plane polarized light (usually the sodium D line, $\lambda = 589$ mμ) is measured on an instrument called a polarimeter and is recorded as specific rotation:

$$\text{Specific rotation} = \frac{\text{observed rotation (degrees)}}{\text{polarimeter tube length (dm)} \times \text{solution concentration (g/ml)}}$$

$$\text{or} \quad [\alpha]_{\lambda}^{T^{\circ}C} = \frac{\alpha}{l \times d}$$

If the rotation of the plane of polarized light is to the right the enantiomer or optical isomer is termed dextrorotatory and given the notation (*d*) or (+). If the rotation is to the left the enantiomer is called levorotatory as indicated by a (*l*) or (-) notation.

Compounds which can rotate the plane of polarized light are said to be optically active. In a compound consisting of two enantiomers the pure enantiomers rotate light equally but in opposite directions.

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[3] Enantiomers may be considered to differ in one chemical property, the rate of reaction with dissymmetric compounds (Barker, 1971), and one other physical property, the absolute molecular configuration about the center of asymmetry.

Therefore a mixture of equal parts of the enantiomers is optically inactive as indicated by a (±) notation and is called a racemic modification (mixture). If, however, the enantiomers are resolved (separated) the compound is then optically active and can rotate the plane of polarized light.

APPENDIX 2

Summary of experimental conditions and results reported in studies of the estrogenic and anti-estrogenic effects of DDT.

Animals	Chemical(s)	Solvent and route	Dose	Probable *o,p'*-DDT administered per day (mg/kg)	Time factor	Reported effect	Reference
cockerels	*p,p'*-DDT	chicken fat s.c.	15 mg/kg-300 mg/kg	---	60-89 days	retarded comb and testis growth	Burlington and Lindeman, 1950
ovariectomized rats	tech. DDT methoxychlor DHDT**	propylene glycol s.c.	15/30/45 mg 15/30/45 mg 10/15/20/25/45 mg	? --- ---	5 doses with 8 hour intervals	none none induced estrus	Fisher *et al.*, 1952
cockerels	tech. DDT	--- feed	3000 ppm	?	12 days	decreased sperm production	Albert, 1962
female rats	*o,p'*-DDT	? sc	4 mg	93	1 dose per day for 3 days	increased uterine wet weight, glycogen and RNA	Bitman *et al.*, 1968
female chickens female Japanese quail	*o,p'*-DDT	olive oil ip	50 mg 5 mg	63 63	1 dose per day for 3 days	increased oviducal wet weight and glycogen	

APPENDIX 2 (Continued)

Animals	Chemical(s)	Solvent and route	Dose	Probable *o,p'*-DDT administered per day* (mg/kg)	Time factor	Reported effect	Reference
immature female rats ovariectomized rats	tech. DDT *o,p'*-DDT tech. DDT	DMSO ip	10 mg/kg 10 mg/kg 50 mg/kg	2 10 10	1 dose 1 dose 1 dose per day for 3 days	increased 37% uterine 74% wet weight ?	Levin *et al.*, 1968
	o,p'-DDT		1 mg/kg 50 mg/kg	1 50		14% increased 49%/151%	Welch *et al.*, 1969
			50 mg/kg	10	1 dose only/1 dose per day for 3 days	uterine 43%/75% wet weight 37% 28%/28%	
immature female rats	methoxychlor *p,p'*-DDT *o,p'*-DDD *m,p'*-DDD *p,p'*-DDD *p,p'*-DDE	DMSO ip	50 mg/kg 50 mg/kg 50 mg/kg	--- ---		no significant effect	
immature female rats	*o,p'*-DDT *o,p'*-DDE *p,p'*-DDT	olive oil sc	0.25 mg 4 mg 4 mg	6 --- ---	1 dose	increased uterine glycogen	Bitman and Cecil, 1970

APPENDIX 2 (Continued)

Animals	Chemical(s)	Solvent and route	Dose	Probable *o,p'*-DDT administered per day* (mg/kg)	Time factor	Reported effect	Reference
Japanese quail	*o,p'*-DDT	olive oil im	5 mg	70	1 dose per day for 4 days	ovary and testis weights not affected	Cooke, 1970
		oral	10 mg	47	3 doses/ wk for 3 wks		
ovariectomized rats	*o,p'*-DDT	corn oil im	100 mg/kg	100	1 dose	increased uterine weight, glycogen and enzymes**	Singhal *et al.*, 1970
immature female rats	*o,p'*-DDT	olive oil oral	10 µg	0.14	1 dose per day for 15 days	no effect	Wrenn *et al.*, 1970
			50 µg	0.69		earlier vaginal patency	
immature female rats	*o,p'*-DDT	olive oil sc	4 mg	93	1 dose	increased uterine weight, water, glycogen and RNA	Cecil *et al.*, 1971a

APPENDIX 2 (Continued)

Animals	Chemical(s)	Solvent and route	Dose	Probable *o,p'*-DDT administered per day* (mg/kg)	Time factor	Reported effect	Reference
immature female rats	*o,p'*-DDT	sesame oil ip	1 mg 2 mg 4 mg	?	1 dose	increased uterine weight	Duby *et al.*, 1971
female mink			1 mg/kg 10 mg/kg	1 10	1 dose per day for 3 days	no effect increased uterine weight	
neonatal female rats	*o,p'*-DDT	propylene glycol and ethanol sc	1 mg	?	days 2, 3 and 4 post par-turition	advanced puberty persistent estrus	Heinrichs *et al.*, 1971
ewes	*o,p'*-DDT	? feed	10 ppm	?	2-9 mos	no effect on uterine parameters	Wrenn *et al.*, 1971a
immature female rats	*o,p'*-DDT	--- feed	1 ppm 10 ppm 20 ppm 40 ppm	?	age 21 days to 6, 13 or 17 mos	vaginal patency unaffected	Wrenn *et al.*, 1971b
		olive oil oral	50 µg 200 µg	0.24 1.0	1 dose per day for 14 days	general reproduction unaffected	

APPENDIX 2 (Continued)

Animals	Chemical(s)	Solvent and route	Dose	Probable *o,p'*-DDT administered per day (mg/kg)	Time factor	Reported effect	Reference
immature female rats	*o,p'*-DDT	ethanol (evap.) feed	500 ppm	50	age 23-30 days	earlier vaginal patency	Clement and Okey, 1972
			1000 ppm	100		earlier vaginal patency and increased uterine wet and dry weight and glycogen	
			2000 ppm	200			
			3000 ppm	300			
immature female rats	*o,p'*-DDT	DMSO ip	500 µg	3.2	1 dose per day for 27 days	earlier vaginal patency and increased ovarian and uterine weight	Gellert *et al.*, 1972
ovariectomized rats	*o,p'*-DDT		10 mg	46	1 dose per day for 7 days	increased uterine weight	
	p,p'-DDA		10 mg	---			
mature female mice	*p,p'*-DDT	peanut oil ip	40 mg/kg	---	1 dose	prolonged estrus cycle	Örberg *et al.*, 1972

APPENDIX 2 (Continued)

Animals	Chemical(s)	Solvent and route	Dose	Probable *o,p'*-DDT administered per day* (mg/kg)	Time factor	Reported effect	Reference
mature male mice	tech. DDT	corn oil oral	12.5/25/ 50 mg/kg	2.5/5/10	1 dose per day for 10 days	inhibited uptake of testosterone by prostates	Smith *et al.*, 1972
ovariectomized mice			25 mg/kg	5		no effect	
adult female rats	*o,p'*-DDT	sesame oil ip	90 µg 900 µg	?	1 dose per day for 3 days post coitum	no effect on blastocyst implantation	Van Tienhoven and Duby, 1972
mature female mice	*p,p'*-DDT	arachis oil ip	50/100 mg/kg	---	1 dose day 1 of pregnancy	decrease of implanted ova	Lundberg and Kihlström, 1973
male rats	*o,p'*-DDT	---	10 ppm	---	---	inhibited binding of testosterone to prostate receptor proteins *in vitro*	Wakeling and Visek, 1973

APPENDIX 2 (Continued)

Animals	Chemical(s)	Solvent and route	Dose	Probable *o,p'*-DDT administered per day* (mg/kg)	Time factor	Reported effect	Reference
ovariectomized rats	*o,p'*-DDT	olive oil ip	50 mg/kg	50	1 dose	increased uterine weight	Zeitoun and Makar, 1973
mature female rats	*o,p'*-DDT	ethanol (evap.) feed	1000 ppm	?	6 mos	reproductive failures in F_1 females	Clement and Okey, 1974
neonatal female rats	*o,p'*-DDT	DMSO sc	0.1 mg	?	1 dose per day 2, 3, and 4 post-parturition	advanced puberty persistent estrus	Gellert *et al.*, 1974
neonatal male rats			1.0 mg	?		no effect	
mature male mice	tech. DDT	corn oil oral	12.5/25/50 mg/kg	2.5/5/10	1 dose per day for 10 days	inhibited uptake of testosterone by prostates	Lloyd *et al.*, 1974
mature female rats	*o,p'*-DDT	---	1/5/10 ppm	---	---	inhibited uptake of estradiol by uterine receptors *in vitro*	Nelson, 1974

APPENDIX 2 (Continued)

Animals	Chemical(s)	Solvent and route	Dose	Probable *o,p'*-DDT administered per day* (mg/kg)	Time factor	Reported effect	Reference
neonatal male rats	*o,p'*-DDT	sesame oil ?	2 mg	?	1 dose per day days 1-5 post-parturition	no effect on testes	Campbell and Mason, 1975
immature female rats	*o,p'*-DDT	---	1.4×10^{-4} M	---	---	inhibited uptake of estradiol by uterine receptors *in vitro*	Forster *et al.*, 1975
mature female rats	*o,p'*-DDT	5% ethanol in sesame oil oral	10 mg/day	?	1 dose per day, days 15-19 of pregnancy	no effect on F_1 ovaries or estrus	Gellert and Heinrichs, 1975
mature female mice	*o,p'*-DDT	peanut oil sc	50 mg/kg	---	1 dose per week	reproductive failures in F_1 females only if bred to F_1 males	Kihlström *et al.*, 1975

APPENDIX 2 (Continued)

Animals	Chemical(s)	Solvent and route	Dose	Probable *o,p'*-DDT administered per day* (mg/kg)	Time factor	Reported effect	Reference
neonatal male rats	*o,p'*-DDT	20% ethanol in propylene glycol sc	2.82 µmole	?	1 dose per day, days 2, 3, and 4 post-parturition	no effect on steroid imprinting	Gustafsson and Stenberg, 1976
mature female rats	tech. DDT	feed	150 ppm	?	36 wks	0 of 3 produced progeny	Jonsson *et al.*, 1976

*This calculation, based on the estimated food consumptions, animal weight, or the number of doses administered per day, can be used along with the 'time factor' data for comparisons among studies in which sufficient data were supplied.

**See text.

APPENDIX 3

Literature review for (±)-*o,p'*-DDD

It has been known for some time that (±)-*o,p'*-DDD possesses adrenocorticolytic activity in mammalian systems (Straw and Hart, 1975) and the following review contains a summary of the literature pertinent to this area of research.

The adrenal cortex

The mammalian adrenal cortex is an endocrine organ which secretes (1) glucocorticoids, steroids affecting carbohydrate and protein metabolism, (2) aldosterone, a mineral corticoid which regulates sodium balance, and (3) small amounts of steroids with sexual activity. The adult adrenal cortex consists of an outer zona glomerulosa, a middle zona fasciculata and an inner zona reticularis (Ganong, 1969). These three zones may be of variable distinctness with regard to both histology and ultrastructure (Luse, 1967; Ganong, 1969).

Aldosterone (Figure 16) is produced only in the zona glomerulosa while the glucocorticoids and sex hormones can be produced in all three adrenal cortical tissue layers (Ganong, 1969). The most active glucocorticoid produced by humans is cortisol (Figure 16) and its production is controlled through a feedback mechanism with pituitary ACTH (adrenocorticotropic hormone) (McGilvery, 1970). In rodents, however,

corticosterone is the major adrenal cortical secretory product and the cortisol to corticosterone ratio varies among species (Samuels and Uchikawa, 1967).

A large number of steroid precursors, intermediates and metabolites as well as the active compounds themselves are usually present in the plasma or urine commonly assayed as a measure of adrenal cortical activity. Comprehensive reviews of the biochemistry of the adrenal cortex have been published (Cope, 1972; Eisenstein, 1967) and a brief summary from them will provide a basis for the interpretation of the findings for the (±)-*o,p'*-DDD studies below.

Most adrenal steroids except for the androgens and some metabolites are C_{21} steroids. Their biosynthesis begins with the production of pregnenolone from either cholesterol or acetate (Cope, 1972) (Figure 16). Pregnenolone is acted on by various oxidase, isomerase and hydroxylase enzymes in the adrenal cortex to produce cortisol, corticosterone and aldosterone via progesterone (Cope, 1972; Samuels and Uchikawa, 1967) (Figure 16).

Most of the functional steroids have half lives measured in terms of minutes only and are broken down principally in the liver (Cope, 1972). Reductions and hydroxylations of cortisol result in such metabolites as those shown in Figure 17.

For dissolution and transport in plasma, cortisol and corticosterone are bound to a globulin protein called transcortin or CGB (corticosteroid binding globulin) while the excretion of the above metabolites is dependent on an increased water solubility resulting from conjugation with glucuronic acid (Cope, 1972).

The adrenal cortex also produces significant levels of androgens such as dehydroepiandrosterone and its sulfate, and probably trace

Figure 16. Biosynthesis of cortisol and aldosterone (Cope, 1972).

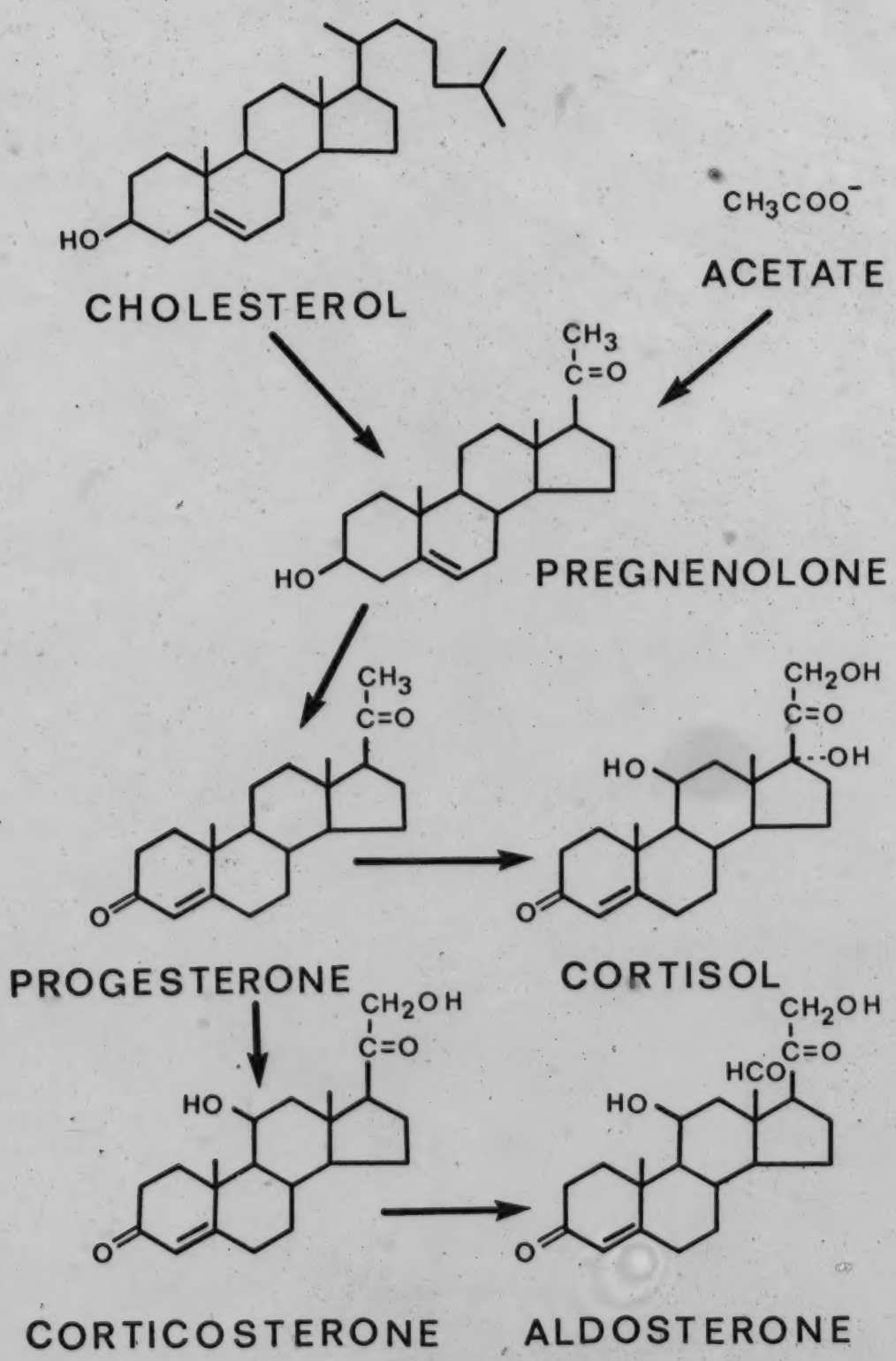
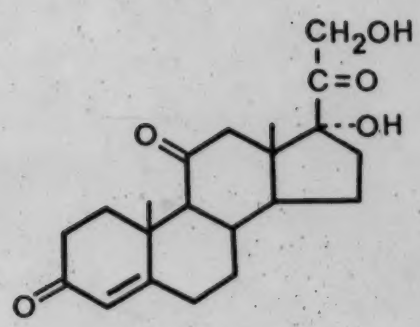
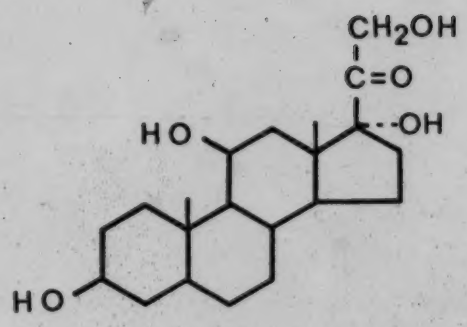

HO
CHOLESTEROL
CH3COO-
ACETATE
CH3
C=O
HO
PREGNENOLONE
CH3
C=O
O
PROGESTERONE
CH2OH
C=O
HO
OH
O
CORTISOL
CH2OH
C=O
HO
O
CORTICOSTERONE
CH2OH
C=O
HCO
HO
O
ALDOSTERONE

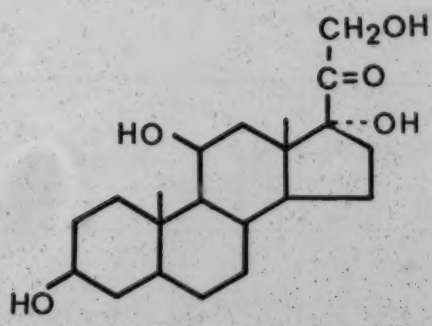
Figure 17. Some common metabolites of cortisol (Cope, 1972).



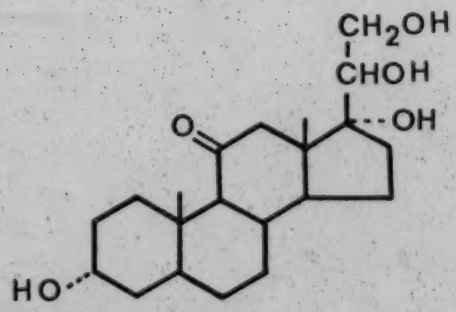
CORTISONE



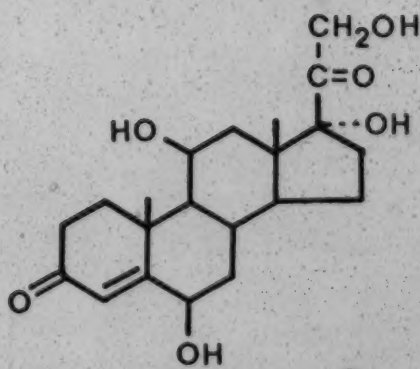
TETRAHYDROCORTISOL



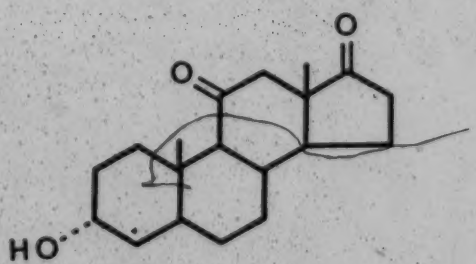
CORTOL



CORTOLONE



6ß-HYDROXYCORTISOL



3∝-HYDROXYAETIOCHOLANE-11,17-ONE

amounts of estrogens but normally this production is minor compared to the production of these steroids by other organs (Cope, 1972).

o,p'-DDD-early studies

In 1949, Nelson and Woodard reported that technical grade TDE (DDD) had produced severe adrenal cortical atrophy in 10 dogs treated with 50 - 200 mg/kg/day for 1 - 33 months. Grossly, the affected adrenals were reduced in size and microscopic examination revealed that the zona reticularis had atrophied totally. The zona fasiculata exhibited cellular changes and structural disorganization while the least affected zona glomerulosa showed some cellular and zonal outline changes. A later study revealed that in extreme cases necrosis of the entire adrenal cortex of the dog could be produced (Bergenstal *et al.*, 1960) by DDD treatment. The adrenal medulla was unaffected. On this basis it was concluded that an example of high "chemical specificity in the causation of damage of an organ" had been discovered and the similarity of this damage to that seen for Addison's disease (adrenal hypocorticoidism) in man was recognized (Nelson and Woodard, 1949).

Previous DDD studies on rats, mice, rabbits as well as dogs had not resulted in the above-mentioned adrenal damage (Nelson and Woodard, 1949). Similarly, in the first reported controlled DDD treatment of man no effect on the adrenals was observed (Sheehan *et al.*, 1953). Two factors probably were responsible for these findings: most importantly the "active factor" of technical grade DDD had not been isolated and may not have been present in all preparations of the technical grade DDD used; secondly, a difference in species response to DDD has been found to exist (Foster, 1968; Nichols and Hennigar, 1957).

The reason for DDD being administered to man by Sheehan *et al.* (1953) was to investigate the possibility that it could cure Cushing's syndrome (cortisol overproduction (Cope, 1972)) through its above-described effects on the adrenal cortex. Although the DDD treatment was a logical one it failed to offset the effects of Cushing's syndrome probably because of the low levels administered (1.06 g/day for 18 days, none for 39 days and then 2.84 g/day for 30 days) and possibly because of the absence of the necessary "active factor."

The first study to isolate this "active factor" from technical grade DDD resulted in the identification of *o,p'*-DDD as at least partly responsible for the observed adrenal atrophy in dogs (Nichols and Hennigar, 1957). It was possible to extract the "active principle" from the fat and adrenals of treated rats (the rat adrenals were apparently unaffected by the DDD treatment) but this extraction was not possible from the fat and adrenals of dogs with atrophied adrenals. The *o,p'*-isomer of DDD was confirmed as the major adrenocorticolytic agent in technical grade DDD by Cueto *et al.* (1958) while *p,p'*-DDD was shown to be inactive. By colorimetric analysis these authors showed that *o,p'*-DDD did accumulate in the adipose and adrenal tissues of dogs suggesting that Nichols and Hennigar (1957) may have observed only a difference in the affinity of *o,p'*-DDD for the tissues of dogs and rats.

Besides observing the general health and presence or absence of adrenal atrophy a more sensitive test was used by Cueto *et al.* (1958) to measure adrenocorticolytic activity. ACTH was administered and a positive response to DDD was indicated by a failure of the level of blood eosinophils to drop and a failure of the urinary excretion of 17-hydroxycorticoid steroids to increase. These authors emphasized

the importance of determining the site of action of the *o,p'*-DDD questioning which of pituitary production of ACTH, adrenal response to ACTH or adrenal steroid synthesis was being affected.

In 1962, Cazorla and Moncloa reported an *in vitro* study to elucidate the mode of action of *o,p'*-DDD on the dog adrenal cortex. They found a decreased adrenal response to ACTH and a partial inhibition of glucose-6-phosphate dehydrogenase (G-6-PD). They felt that the inhibition of this enzyme could explain the decreased production of adrenal 17-ketosteroids since the synthesis of steroids requires reduced triphosphopyridine nucleotide (NADPH), a product of the G-6-PD enzyme (Srebočan *et al.*, 1971).

o,p'-DDD-metabolic effects

Gallagher *et al.* (1962) confirmed a decrease in hydroxycortisone metabolites in man treated with *o,p'*-DDD and as well a decrease in androgen metabolites. But although pregnane-3α, 17, 20α-triol also decreased and was a sensitive indicator of the effect of *o,p'*-DDD in man, Δ^5-pregnane-3β, 17, 20α-triol remained at high levels suggesting an alteration in the peripheral transformations of these steroids. A follow-up study confirmed that *o,p'*-DDD decreased the transformation of 3β, 17-dihydroxy-Δ^5-pregnane-20-one 7α-H^3 to pregnane-3α-17, 20α-triol but did not inhibit its conversion to Δ^5-pregnane-3β, 17, 20α-triol (Bradlow *et al.*, 1963). Inhibition of 3β-hydroxy-Δ^5-steroid dehydrogenase was suggested as the reason for this difference.

This altered peripheral steroid metabolism was confirmed by Bledsoe *et al.* (1964) who found that the previously measured decrease in urinary hydroxycorticosteroids following *o,p'*-DDD treatment might not have

reflected a decreased adrenal production of steroids since they found that cortisol was preferentially converted to 6β-hydroxycortisol in their patients while plasma levels and adrenal production of cortisol remained stable. The 6β-hydroxycortisol was more difficult to extract from urine than the normally excreted tetrahydrocortisol and tetrahydrocortisone and failure to measure its presence would give misleading low levels of urinary steroids. It was suggested by Kupfer *et al.* (1964) in a study using guinea pigs that *o,p'*-DDD might have stimulated the liver microsomes in their animals to effect the observed alterations in steroid metabolism.

An *in vitro* study confirmed that *o,p'*-DDD could stimulate the metabolism of cortisol by liver microsomes (9000-g supernatant) from rats, pretreated with the chemical. A similarity of drug-metabolizing and steroid metabolizing enzymes had been noted previously and an increase in liver weights was thought to have been indicative of a *de novo* synthesis of these enzymes (Kupfer and Peets, 1966). But such reports do not contradict the earlier findings that prolonged *o,p'*-DDD treatment could cause a decrease in plasma steroids and affect adrenal carcinoma metastases (Bledsoe *et al.*, 1964; Cope, 1972).

Kupfer (1969) in a review of the literature to that time again concluded that *o,p'*-DDD was effective as an inducer of liver microsomes as related to steroid metabolism in both rats and guinea pigs. Technical grade DDT was a more potent microsomal inducer than *p,p'*-DDT but it contained 20% *o,p'*-DDT which could in part have been metabolized to *o,p'*-DDD (Feil *et al.*, 1973) before acting.

Recently, it was shown that *o,p'*-DDT administered to dogs was a more potent microsomal inducer than *o,p'*-DDD and the portion of

o,p'-DDT metabolized to *o,p'*-DDD in the liver was not accumulated sufficiently in the adrenals to exert an adrenocorticolytic effect (Copeland and Cranmer, 1974). The induced increase of hydroxylation of cortisol to 6β-hydroxycortisol did cause an increased adrenal size probably because of the altered feedback between cortisol and ACTH.

In mice a single dose of *o,p'*-DDD caused an increase in pentobarbital-induced sleeping time indicating a possible competition for certain liver enzymes (Gabliks and Maltby-Askari, 1970) but chronic treatment with *o,p'*-DDD led to a normal pentobarbital response suggesting that an additional enzyme complement had been synthesized. This diphasic response could possibly explain the later findings that *o,p'*-DDD was apparently ineffective in inducing microsomal mixed function oxidases in mice (Abernathy *et al.*, 1971).

It has also been shown that *o,p'*-DDD caused altered androgen metabolism in man (Hellman *et al.*, 1973) and an inhibition of steroid hydroxylation in both man and dogs (Brown *et al.*, 1973). While this latter study and that of Jarabak and Street (1971) indicated that only 11β-hydroxylation was inhibited Komissarenko *et al.*, (1972b) found evidence for 3β- and 21-hydroxylase inhibition as well.

A review of the effects of environmental chemicals on steroid metabolism has been published recently (Conney *et al.*, 1973) and these authors emphasized that in rats the same hepatic enzyme system hydroxylates both steroids and some drugs. One of the potent stimulators of rat microsomal enzymes is *o,p'*-DDD which also stimulates 6β-hydroxylation of steroids in man. Obviously it may be concluded that *o,p'*-DDD has the ability to induce liver microsomal enzymes and thereby alter peripheral steroid and drug metabolism.

o,p'-DDD-clinical investigations

Bergenstal *et al.* (1960) were the first to use the adrenocorticolytic property of *o,p'*-DDD to treat metastatic adrenocortical carcinoma, a relatively rare cancer occurring in about two per million population (Hutter and Kayhoe, 1966a). They administered 8-10 g *o,p'*-DDD/day for 4-8 weeks to eighteen patients. The metastases regressed in seven treated patients, the urinary steroid levels (17-hydroxycorticoids and 17-ketosteroids) dropped in another seven and four were unaffected. These authors considered the results of the treatment encouraging but because of the toxicity of *o,p'*-DDD previously described by Sheehan *et al.* (1953) (gastrointestinal, central nervous system, dermal and muscular effects) they suggested development of an effective but less toxic analog. Man, it seemed from this study, was less sensitive to *o,p'*-DDD than the dog but individual variation in responses among the patients was marked. This variability could not be explained by the variation in metabolism of *o,p'*-DDD found in the patients (Moy, 1961). From 60 to 65% of the *o,p'*-DDD dose was not absorbed from the gut and was excreted together with another 25% which had been metabolized to a water soluble derivative. Moy (1961) suggested that the water soluble metabolite was *o,p'*-DDA and this was confirmed (Sinsheimer *et al.*, 1972).

It was reported later than *o,p'*-DDA as well as several hydroxylated forms of *o,p'*-DDA were metabolites of *o,p'*-DDD which could be extracted from the urine of treated humans and from the urine and feces of treated rats (Reif and Sinsheimer, 1975; Reif *et al.*, 1974). Bovine adrenal glands were able to convert *o,p'*-DDD to *o,p'*-DDA in an *in vitro* study (Reif *et al.*, 1975) and the conversion of *o,p'*-DDD to *o,p'*-DDE

has been shown for humans (Kvalvåg and Stenersen, 1974).

Hutter and Kayhoe (1966a,b) published the results of an extensive survey of the effects of *o,p'*-DDD on adrenal cortical carcinoma. They reported the findings of 105 doctors who treated 138 patients with adrenal carcinoma between 1960 and 1965 under the direction of the NCI (National Cancer Institute). A further 48 published cases were also reviewed. Daily doses of 8-10 g resulted in the same reversible toxic effects found earlier (Bergenstal *et al.*, 1960) but a greater than 50% reduction in urinary hormone excretion occurred in 43 of 62 patients (69%) while 20 of 59 (34%) showed "objective tumor regression." Therefore from these findings it was concluded that "*o,p'*-DDD is indicated in the treatment of inoperable patients with both functioning and non-functioning adrenal carcinoma" since it may have increased patient longevity.

Temple *et al.* (1969) while treating hypercorticoidism caused by Cushing's syndrome were able to reduce the side effects of *o,p'*-DDD by lowering the daily dosage (3 g) and extending the administration period. They found a decreased production of 17-hydroxycorticoids and hydrocortisones but an unaltered production of aldosterone. Therefore *o,p'*-DDD definitely did not alter peripheral steroid metabolism only but gave some hope as an effective chemotherapeutic agent in the treatment of hypercorticoidism (Ingelfinger, 1969). The altered steroid production in one patient was correlated with altered cristae of the mitochondria of the zona fasciculata and an essentially unchanged zona glomerulosa as previously reported for dogs (Temple *et al.*, 1969).

Two further simultaneous reports on *o,p'*-DDD treatment of adrenocortical carcinoma gave sightly different results. Sakauchi *et al.*,

(1969) found regression of the metastases anddecreases in urinary 17-ketosteroids, 17-hydroxycorticosteroids, 17-ketogenic steroids and estrogens as well as plasma 17-hydroxycorticosteroids and cortisol. Bochner *et al.* (1969) reported a regression of metastases but found no decrease in urinary 17-hydroxycorticosteroids, the only measured steroids. Each study involved only one patient.

On the basis of the many above findings the FDA (United States Food and Drug Administration) in 1970 approved *o,p'*-DDD for use in the treatment of adrenocortical carcinoma and Cushing's syndrome (Hoffman and Mattox, 1972; Sinsheimer *et al.*, 1972). The mode of action of *o,p'*-DDD, however, was still only poorly understood.

In two *in vitro* studies using excised dog adrenals treated with *o,p'*-DDD *in vivo* it was shown that *o,p'*-DDD and not one of its metabolites was in fact the active compound (Hart and Straw, 1971a). Furthermore, *o,p'*-DDD blocked the ACTH-induced intramitochondrial conversion of cholesterol to pregnenolone but did not affect the baseline steroidogenesis (Hart and Straw, 1971b). A temporal relationship between the effects of DDD on ACTH-induced steroid output in dogs and the ultra- and histological structure of the adrenal cortex was also found (Hart *et al.*, 1973). Surprisingly, although *m,p'*-DDD was known from 1961 to have an effect similar to that of *o,p'*-DDD (Temple *et al.*, 1969) this study demonstrated that it was in fact the more potent of the two in inhibiting ACTH-induced steroid production and producing ultrastructural and histological damage. This is in contrast to the report by Reznikov (1973) that *o,p'*-DDD was a more effective cortico-lytic agent than *m,p'*-DDD. The *p,p'*-isomer of DDD also caused a decreased ACTH-induced steroid production although it required

considerably more time than the other two isomers to do so (Hart *et al.*, 1973).

Young *et al.* (1973) reported that *o,p'*-DDD appeared to interfere with adrenal steroid synthesis by inhibiting the 11β- and 21-hydroxylation as well as side chain cleavage of cholesterol. These inhibitions were thought to be the result of an association of the *o,p'*-DDD with microsomal and mitochondrial cytochrome P-450. Martz and Straw (1976) later determined that the P-450 cytochrome was decreased by *o,p'*-DDD treatment and concomitant reductions in microsomal protein and heme also occurred. These latter authors theorized that the *o,p'*-DDD acts directly by destroying the protein, heme and P-450 and thereby eliminating steroid biosynthesis.

Komissarenko *et al.* (1972a) reported that normal canine adrenal structure and function were only partially restored after nine months of recovery time following treatment with *o,p'*-DDD. Kirk and Jensen (1975) found that the zona fasciculata and zona reticularis of normal dogs could be destroyed with only one to two weeks of treatment with 50 mg/kg of *o,p'*-DDD but that 8 of 10 dogs treated in this manner appeared healthy after 16 weeks of treatment. This sensitivity of dogs to *o,p'*-DDD makes them an excellent test animal for studies of *o,p'*-DDD and indicates that *o,p'*-DDD is an effective drug for use in the treatment of canine hyperadrenocorticoidism (Lorenz *et al.*, 1973; Schecter *et al.*, 1973).

Recently three reviews of the treatment of human adrenocortical carcinoma with *o,p'*-DDD have been published (Hoffman and Mattox, 1972; Lubitz *et al.*, 1973; Straw and Hart, 1975). In the first of these it was concluded that *o,p'*-DDD given to 18 patients was not as effective

as the earlier reports cited would have indicated. They found toxicity to be a major deterrent to its use and emphasized that because of the large number of variables involved with diseased patients it was not possible to predict who would respond to the treatment. However, it was recommended that *o,p'*-DDD be administered in cases where either surgery was not possible or where lowered adrenocortical hormone levels would be beneficial. Hajjar *et al.* (1975) presented data similar to those of Hoffman and Mattox (1972) and concluded that "there is no convincing evidence that *o,p'*-DDD has materially prolonged the lives of patients suffering from adrenal cancer."

Alternatively, Lubitz *et al.* (1973) in a paper summarizing the findings in 115 adrenocortical carcinoma patients treated since the report of Hutter and Kayhoe (1966a;b) concluded that *o,p'*-DDD was more effective than indicated in earlier reports. They reported that 85% of their patients showed a 30% or greater decrease in urinary steroids, 61% a "measurable disease response" (reduction in tumor size) and 45% a "clinical response" (overall health improvement). Continued early treatment with *o,p'*-DDD in conjunction with steroid replacement therapy was recommended for optimal control of the disease. Straw and Hart (1975) presented a detailed literature review of the effects of *o,p'*-DDD in vertebrate systems and discussed the same data which had appeared in the report by Lubitz *et al.* (1973).

A comment in the 1973 report of Lubitz *et al.* that no one had been "cured" by *o,p'*-DDD resulted in a letter to the editor in which it was reported that a patient with metastatic adrenal carcinoma treated with mitotane (*o,p'*-DDD) and fluorouracil had been "cured" for 7 years (Schick, 1973). Similar "cures" were reported by Becker and Schumacher

3 3

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(1975) and Ostuni and Roginsky (1975). Sizonenko *et al.* (1974) also reported successful treatment of a patient suffering from bilateral adrenal hyperplasia and Cushing's syndrome using *o,p'*-DDD. Luton *et al.* (1973) obtained encouraging results in the *o,p'*-DDD treatment of Cushing's syndrome when all symptoms except osteoporosis disappeared in 6 of 17 patients while 10 of the 17 had complete hypercortisolism regression 12 to 32 months after the drug was withdrawn. Scott (1975) recommended early treatment of inoperable adrenocortical carcinoma patients with a combination of *o,p'*-DDD and aminoglutethimide indicating that *o,p'*-DDD has not lost favor in the treatment of this disease.

Recently, it has been reported that *o,p'*-DDD-^{131}I has given excellent results as a radiopharmaceutical for use in the scanning of adrenal glands for tumors (Skromme-Kadlubik *et al.*, 1975). The authors cautioned that the drug must be used taking into account the circadian rhythmicity of adrenal activity.

o,p'-DDD-other studies

Several papers dealing with miscellaneous side effects of treatment with *o,p'*-DDD and its analogs have been published. In the chick *o,p'*-DDD interfered with vitamin D metabolism and caused histologically determined adrenal damage (Chen and Bosman, 1967). Also in chickens technical grade DDT and *o,p'*-DDT were reported to be effective inhibitors of corticosterone production (Srebočan *et al.*, 1971) although the possibility that this effect was a function of an altered steroid metabolism alone cannot be excluded. Chronic administration of *o,p'*-DDD to rats caused increased thyroid weights probably through an induced increase in thyroxine metabolism resulting in a compensated

hypothyroidism (Fregly *et al.*, 1968). Also in rats *o,p'*-DDD prevented renal hypertension and the associated effects of bilateral encapsulation of the kidneys with latex envelopes but the mode of action was not ascertained (Fregly, 1968). Prolonged treatment of the rat with *o,p'*-DDD caused Leydig cell tumors possibly because of increased androgen metabolism since estrogen treatments were able to cause the tumors as well (Lacassagne, 1971).

An *o,p'*-DDD-induced glucocorticoid deficiency in dogs had few apparent direct circulatory effects except for the ability to withstand the stress of thoracotomy and multiple injections of vasopressor amines (Cueto and Moran, 1968). Dogs were found to absorb more *o,p'*-DDD from the alimentary canal than did guinea pigs (Korpachov, 1972) possibly explaining one species difference in the response to this chemical. Men occupationally exposed to DDT were found to have normal adrenocortical secretion of cortisol (Clifford and Weil, 1972).

Administration of Aroclor 1221 polychlorinated biphenyls (PCB's) to rats resulted in adrenocortical hyperfunction as measured by plasma corticosterone levels (Wassermann *et al.*, 1973). This increase was thought to be a result of stress induced by the PCB's or competitive metabolism between the measured steroid and the PCB's in the liver.

In an *in vitro* study utilizing rat uteri, 10 ppm *o,p'*-DDD was shown to inhibit 17β-^{3}H-estradiol binding to uterine receptors (Nelson, 1974) and in an *in vivo* study this estrogenic activity was confirmed (Gellert and Heinrichs, 1975). Injections of 1 mg (±)-*o,p'*-DDD into neonatal rats on days 2, 3, and 4 after birth resulted in persistent vaginal estrus and an absence of corpora lutea later in life as previously reported following treatment with known estrogens and

(±)-*o,p'*-DDT (Heinrichs *et al.*, 1971).

The crystal structure of *o,p'*-DDD has been reported recently by Arora and Bates (1976).

APPENDIX 4

Experimental

(±)-1-Chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]benzene

9 kg technical grade DDT dissolved in 66 l of hot ethanol was crystallized at room temperature and -15°C yielding predominantly *p,p'*-DDT crystals. The mother liquors were evaporated, the residue dissolved in pentane, seeded with *p,p'*-DDT and crystallized at room temperature and -15°C. The crystals formed were predominantly *p,p'*-DDT. The reduced pentane mother liquors were crystallized at room temperature and -15°C after seeding with *o,p'*-DDT. The resulting crystals were predominantly *o,p'*-DDT and were crystallized 3 times from methanol. The resulting (±)-*o,p'*-DDT (516.9 g, 30.8%) contained about 1.3% *p,p'*-DDT by weight (GLC analysis). *Anal.* Calcd for $C_{14}H_9Cl_5$: C, 47.4; H, 2.6; Cl, 50.0. Found: C, 47.8; H, 2.6; Cl, 49.7. mp 72-74°C; lit. 73-74°C. GLC and TLC typical of *o,p'*-DDT. NMR ($CDCl_3$) δ 5.77 (s, 1H, benzylic), 7.16-7.61 (m, 7H, aromatic), 8.02-8.22 (m, 1H, aromatic).

(±)-1-Chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]benzene-4-sodium sulfonate ((±)-SO_3Na-*o,p'*-DDT)

9.9 g (±)-*o,p'*-DDT was dissolved in 10 ml $CHCl_3$ at 10°C. 3.7 ml of $ClSO_3H$ was added over a period of 5 min and the stirred mixture was

maintained at 12 ± 2°C for a further 85 min. The reaction mixture was poured onto 50 g ice and the resulting emulsion diluted with 100 ml of H_2O and neutralized with $NaHCO_3$. Following filtration, the $CHCl_3$ was removed and the heated solution saturated with NaCl, cooled and filtered. The filtrate was dissolved in 200 ml acetone and filtered to remove any excess NaCl. The filtrate was shaken with 160 ml hexane followed by 160 ml H_2O. The product in the resulting water-acetone layer after a further recrystallization from 10% NaCl in H_2O weighed 7.37 g (58%). 2.26 g of *o,p'*-DDT was recovered from the hexane. *Anal.* Calcd for $Cl_{14}H_8Cl_5SO_3Na$: C, 36.8; H, 1.8; Cl 38.9; S, 7.0. Found: C, 36.7; H, 2.1; Cl, 38.8; S, 6.9. NMR ($CDCl_3$) δ 5.56 (s, 1H, benzylic), 6.60-7.52 (m, 6H, aromatic), 8.60 (s, 1H, aromatic).

Attempted desulfonation of (±)-SO_3Na-*o,p'*-DDT to (±)-*o,p'*-DDT

A control sample of 0.25 g of (±)-SO_3Na-*o,p'*-DDT dissolved in 5 ml H_2O in a test tube was shaken with 2.5 ml hexane. The hexane was extracted, washed with 2.5 ml H_2O, dried with Na_2SO_4 and 2 µl injected directly into the GLC. This confirmed no contaminating *o,p'*-DDT in the sulfonated sample. To a further 2.0 g SO_3Na-*o,p'*-DDT was added 50 ml 37% HCl. The stirred solution was sampled once an hour as the temperature was raised by 10 C° increments over a 40°C-100°C range. Replicate trials using 30% and 70% H_2SO_4 were carried out over an 80°C to the boiling points for these solutions. The latter solution was exposed to a stream of superheated steam (230°C) as well.

(±)-1-Chloro-2-[2,2,2-trichloro-1-(3-nitro-4-chlorophenyl)ethyl]-4-nitrobenzene ((±)-$(NO_2)_2$-*o,p'*-DDT

To 20 g of (±)-*o,p'*-DDT was added 133 ml glacial acetic acid and 133 ml of fuming nitric acid. The reaction mixture was maintained at 100°C for 1 hour, cooled, and poured onto ice. The resulting solid was recrystallized from ethanol yielding 16.9 g (67%) of the dinitro compound. *Anal.* Calcd for $C_{14}H_7Cl_5N_2O_4$: N, 6.3; Found: N, 6.1. mp 144.5-145.5°C; lit. 148-148.5°C. NMR ($CDCl_3$) δ 5.84 (s, 1H, benzylic), 7.52-7.75 (m, 3H, aromatic), 8.10 (d, 1H, J = 2.0 Hz, aromatic), 8.23 d of d, 1H, J = 9.0 Hz, J = 2.5 Hz, aromatic), 9.07 (d, 1H, J = 2.5 Hz, aromatic). TLC same as compound iv of Figure 4.

(±)-1-Chloro-2-[2,2,2-trichloro-1-(3-amino-4-chlorophenyl)ethyl]-4-aminobenzene ((±)-$(NH_2)_2$-*o,p'*-DDT

To 16.5 g (±)-$(NO_2)_2$-*o,p'*-DDT and 49.5 g mossy tin in 115 ml ethanol was added slowly 165 ml of conc. HCl. The solution was kept under reflux in a boiling water bath for 1 hour. An excess of ethanol was added and the solution made basic with 40% NaOH and filtered. The ethanol was evaporated and the resultant aqueous solution extracted with diethyl ether which in turn was evaporated yielding 9.5 g (65.5%) of the (±)-$(NH_2)_2$-*o,p'*-DDT. NMR ($CDCl_3$) δ 3.77 (s, 4H, amine), 5.58 (s, 1H, benzylic), 6.52 (d of d, 1H, J = 8.5 Hz, J = 2.5 Hz, aromatic) 6.75-7.27 (m, 4H, aromatic), 7.35 (d, 1H, J = 3.0 Hz, aromatic).

(±)-1-Chloro-2-[2,2,2-trichloro-1-(3-amino-4-chlorophenyl)ethyl]-4-aminobenzene-(+)-2,3-dihydroxybutanedioc acid salts ((±)-$(NH_2)_2$-*o,p'*-DDT-(+)-tartrate salts)

9.5 g (0.025 mole) of (±)-$(NH_2)_2$-*o,p'*-DDT and 7.4 g (0.050 mole) of (+)-tartaric acid (Aldrich Chemical Co., Inc.) were dissolved in boiling ethanol. The solution was concentrated and left 24 hours at 5°C. 2.6 g of salt crystallized. To obtain the amine the salt was shaken with cold 10% HCl for 15 min and 10% Na_2CO_3 was added slowly until the solution was just basic. The $(NH_2)_2$-*o,p'*-DDT was extracted with diethyl ether.

Deamination of $(NH_2)_2$-*o,p'*-DDT

16 ml of 12N H_2SO_4 and 40 ml of glacial acetic acid were added to 1.6 moles of the $(NH_2)_2$-*o,p'*-DDT and the mixture heated until the DDT derivative dissolved. The stirred solution was cooled to 5°C and 0.32 g of $NaNO_2$ was added. Following 40 min of stirring 25 ml of 50% H_3PO_2 was added and the solution allowed to stand 24 hours at 5°C. The solution was diluted with H_2O and extracted with ether giving a yield of about 50% *o,p'*-DDT confirmed by TLC and GLC analyses. $[\alpha]^{RT}_{546} = -2.9°$. The salts of the mother liquor yielded an amine which when deaminated to *o,p'*-DDT had $[\alpha]^{RT}_{546} = 2.1°$.

(±)-1-Chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]-4-nitrobenzene

50 g of (±)-*o,p'*-DDT was dissolved in 500 ml of CCl_4, the solution cooled to 1 ± 1°C and 1.5 ml of conc. H_2SO_4 added. This solution was added to a 1 ± 1°C solution of 112 ml 90% HNO_3 in 500 ml of acetic anhydride previously mixed for 30 min at room temperature. The reaction,

monitored on TLC, was stopped at about 2 hr by pouring onto 3 l of ice. 500 ml 40% NaOH was added and the aqueous phase extracted with pentane. The CCl_4 phase was evaporated and the residue dissolved in ethanol. The crude product was crystallized from ethanol twice and the pure product fractionally collected from an acid-washed alumina column eluted with 20% diethyl ether in hexane or petroleum ether (bp 38-47°C) alone. The yield of pure (±)-NO_2-*o,p'*-DDT recrystallized from ethanol was 22.1 g (39.3%). 14.3 g of (±)-*o,p'*-DDT was recovered. *Anal.* Calcd for $C_{14}H_8Cl_5NO_2$: C, 42.1; H, 2.3; Cl, 44.4; N, 3.5. Found: C, 41.9; H, 2.1; Cl, 44.2; N, 3.3. mp 138-140°C. NMR ($CDCl_3$) δ 5.78 (s, 1H, benzylic), 7.26-7.67 (m, 5H, aromatic), 8.17 (d of d, 1H, J = 9.0 Hz, J = 3.0 Hz, aromatic), 9.09 (d, 1H, J = 2.5 Hz, aromatic).

Proposed (±)-1-Chloro-2-[2,2,2-trichloro-1-(3-nitro-4-chlorophenyl) ethyl]benzene. (Compound ii of Figure 4)

The proposed compound was isolated from the acid-washed alumina column eluted with petroleum ether (bp 38-47°C). NMR ($CDCl_3$) δ 5.85 (s, 1H, benzylic), 7.21-7.69 (m, 6H, aromatic), 8.04-8.30 (m, 1H, aromatic), 8.13 (d, 1H, J = 2.0 Hz, aromatic).

Proposed (±)-1-Chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]-6-nitrobenzene. (Compound iii of Figure 4)

The proposed compound was isolated from the acid-washed alumina column eluted with petroleum ether (bp 38-47°C). NMR ($CDCl_3$) δ 5.84 (s, 1H, benzylic), 7.21-7.78 (m, 6H, aromatic), 8.38 (d of d, 1H, J = 8.0 Hz, J = 2.0 Hz, aromatic).

(±)-1-Chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]-4-aminobenzene

20 g of (±)-NO_2-*o,p'*-DDT, 20 g of mossy tin and 70 ml of ethanol in a round bottomed flask fitted with a condenser were placed under reflux for 10 min in a boiling water bath. 100 ml HCl was added slowly and the mixture maintained under reflux with stirring for 1 hr. The ethanol was removed by evaporation and 800 ml of H_2O, 800 ml of $CHCl_3$ and 200 ml of 40% NaOH were added and shaken. The $CHCl_3$ was removed, the solution twice extracted with diethyl ether, and the ethereal extract dried with Na_2SO_4, filtered and used directly in salt formation. The amine for analysis was crystallized from petroleum ether (bp 38-47°C). *Anal.* Calcd for $C_{14}H_{10}Cl_5N$: C, 45.5; H, 2.7; Cl, 48.0; N, 3.8. Found: C, 45.5; H, 2.9; Cl, 48.0; N, 3.5. mp 108-111°C. NMR ($CDCl_3$) δ 3.68 (s, 2H, amine), 5.67 (s, 1H, benzylic), 6.52 (d of d, 1H, J = 8.5 Hz, J = 2.5 Hz, aromatic), 7.05-7.60 (m, 6H, aromatic).

(±)-1-Chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]-4-aminobenzene-(+)-7,7-dimethyl-2-oxo-bicyclo[2.2.1]heptane-1-methanesulfonate salt

For the original resolution of the (-)-NH_2-*o,p'*-DDT via fractional crystallization, 23.1 g (0.06 mole) of (±)-amine was combined with 15.2 g (0.07 mole) of (+)-7,7-dimethyl-2-oxo-bicyclo[2.2.1]heptane-1-methanesulfonic acid ((+)-10-camphorsulfonic acid) in CH_3CN. For the resolution of (-)-NH_2-*o,p'*-DDT and (+)-NH_2-*o,p'*-DDT by the reflux technique approximately 54.1 g (0.15 mole) of (±)-amine in diethyl ether was combined with 34.0 g (0.15 mole) of (+)-10-camphorsulfonic acid in CH_3CN, the solvents evaporated and the salts (mp 177-183°C with decomposition) redissolved in 2 l of CH_3CN.

(-)- and (+)-NH_2-*o,p'*-DDT-(+)-10-camphorsulfonate salts

The reflux technique described in the text gave 12.4 g (14.1%) of (-)-NH_2-*o,p'*DDT salt, mp 297-298°C with decomposition, and 7.6 g (8.6%) (+)-NH_2-*o,p'*-DDT salt, mp 288-290°C with decomposition.

(-)- and (+)-NH_2-*o,p'*-DDT

11.0 g of (-)-amine salt was dissolved in a small volume of hot ethanol, cooled and an excess of 10% HCl added. The resulting solution was stirred, heated to about 70°C, cooled, made basic with Na_2CO_3 and extracted with diethyl ether. The extract was dried with Na_2SO_4, filtered and evaporated. 6.4 g of (+)-amine salt was treated similarly.

For the (-)-NH_2-*o,p'*-DDT, $[\alpha]_D^{25} = -170.1°$ (c = 0.55, ethanol) while for the (+)-NH_2-*o,p'*-DDT, $[\alpha]_D^{25} = 166.4°$ (c = 0.75, ethanol).

(-)- and (+)-*o,p'*-DDT

200 ml 12N H_2SO_4 and 400 ml glacial acetic acid, heated to about 70°C, were added to about 6.8 g of (-)-NH_2-*o,p'*-DDT. The amine-hydrogen sulfate dissolved and the solution was cooled to 1±1°C. 1.83 g $NaNO_2$ in a small volume of water was added slowly with stirring and the solution stirred another 1 hr at 1±1°C. 336 ml 50% H_3PO_2 was added, stirred 15 min and left at 5±2°C for 72 hr. 675 ml H_2O plus 600 ml 40% NaOH were added and the solution extracted with pentane. The pentane extract was evaporated and eluted from an acid-washed alumina column using petroleum ether (bp 38-47°C). The (-)-*o,p'*-DDT weighed 5.93 g (91.4%). *Anal.* Calcd for $C_{14}H_9Cl_5$: C, 47.4; H, 2.6; Cl, 50.0. Found: C, 47.1, H, 2.6; Cl, 50.3. mp 73.5-75°C.

Similar ratios of reactants were used to deaminate the (+)-NH_2-*o,p'*-DDT. The (+)-*o,p'*-DDT weighed 3.36 g (89.8%). *Anal.* Calcd for $C_{14}H_9Cl_5$: C, 47.4; H, 2.6; Cl, 50.0. Found: C, 47.5; H, 2.5; Cl, 50.5. mp 73-75°C.

Both enantiomers were crystallized from ethanol and TLC, GLC and NMR analyses for both were typical of racemic *o,p'*-DDT. The specific rotations were $[\alpha]_D^{25} = -17.9^\circ$ (c = 5.05, ethanol) and $[\alpha]_D^{25} = 17.7^\circ$ (c = 2.22, ethanol). The 1.3% *p,p'*-DDT contamination in the starting material was eliminated during the resolution process.

(±)-1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]-4-hydroxybenzene

4.0 g of (±)-NO_2-*o,p'*-DDT was diazotized (as above). The resulting solution of the salt in acetic acid and H_2SO_4 was added slowly with equal volumes of conc. H_2SO_4 to a 135°C mixture of 120 ml H_2SO_4 plus 80 ml H_2O. The solution was stirred at 135°C for 15 min and poured onto 2 l ice. This solution was extracted with pentane and $CHCl_3$, the extracts combined and evaporated. The crude extract weighed 4.4 g (105%) and gave 6 spots on TLC (silica gel G developed with 5% ethylacetate in benzene). Elution with pentane from a silicic acid-celite (3:1) column yielded 0.55 g of the final product. NMR ($CDCl_3$) δ 5.47 (s, 1.3H, hydroxyl), 5.70 (s, 1H, benzylic), 6.78 (d of d, 2H, J = 10.0 Hz, J = 3.0 Hz, aromatic), 7.23-7.63 (m, 7H, aromatic), 7.73 (d, 1H, J = 3.0 Hz, aromatic). Mass spectrum ((±)-OH-*o,p'*-DDT) m/e 368 (5Cl, 1.5%, M^+), 333 (4Cl, 1.8%, M-Cl), 297 (3Cl, 1.8%, M-HCl_2), 262 (2Cl, 5.8%, M-HCl_3), 251 (2Cl, 100%, M-CCl_3), 215 (1Cl, 5.5%, M-$HCCl_4$). Mass spectrum (OH-*o,p'*-DDE) m/e 334 (4Cl, 100%, M+2), 332 (4Cl,

70.9%, M+.), 297 (3Cl, 10.3%, M-Cl), 262 (2Cl, 56.4%, M-Cl_2), 261 (2Cl, 8.8%, M-Cl_2, TFA), 233 (2Cl, 51.3%). To facilitate comparisons among spectra the m/e ratios for the above two compounds have been corrected for the mass of the TFA derivatizing agent. This allows a direct comparison to the data for another (±)-OH-*o,p'*-DDT derivative (Feil *et al.*, 1973) and by a simple correction to the data for a MeOH-*o,p'*-DDE derivative (Feil *et al.*, 1975).

(±)-2-chloro-α-(4-chlorophenyl)benzeneacetic acid

To 5 g (±)-*o,p'*-DDT in 40 ml diethyleneglycol was added 8.8 g KOH in 5 ml H_2O. The mixture was kept under reflux with stirring at 135°C for 6 hours, cooled and poured with vigorous stirring into 100 ml of cold water. The resultant solution was extracted twice with ether, acidified to excess with 20% H_2SO_4 and again twice extracted with ether. The first ether extract was evaporated to dryness and recovered *o,p'*-DDE (1.3 g) was recrystallized from ethanol. The dried second ethereal extract was recrystallized from benzene and gave 1.9 g (47.9%) (±)-*o,p'*-DDA. mp 104-105°C; lit. 106-107°C. NMR ($CDCl_3$) δ 5.49 (s, 1H, benzylic), 7.14-7.34 (m, 8H, aromatic), 9.10 (s, 1H, hydroxyl).

APPENDIX 5

The modified cleanup method of Langlois-Stemp-Liska (Bonelli, 1966) used for the extraction of *o,p'*-DDT residues from the Japanese quail feed samples

1. 60/100 mesh Florisil (Fisher Scientific Co.) previously activated at 1200°C was reactivated at 150°C for a minimum of 12 hours in a loosely capped quart sealer.
2. The Florisil was partially deactivated with 3% redistilled water by weight and kept in an airtight rubber-sealed quart sealer until used.
3. 50 g of Florisil was placed in the column and prewashed with 100 ml of a 50:50 methylene chloride:redistilled petroleum ether (bp 38-47°C) (Fisher Scientific Co.) solution.
4. 1 g of the sample was added to an additional 50 g of Florisil and ground in a mortar until a free-flowing powder resulted. This was added to the top of the column above the previous 50 g of Florisil.
5. Two consecutive 100 ml portions of a 1:4 mixture of methylene chloride:petroleum ether were used to rinse the glassware and utensils and along with a further 400 ml of this mixture were added to the reservoir and allowed to elute from the column.
6. The eluate was flash evaporated to dryness using a Büchi rotary evaporator and a water bath maintained at 35-45°C.

7. 10 ml of spectroanalyzed hexanes (Fisher Scientific Co.) was added to the flask and the resulting solution placed in a labelled culture tube.

APPENDIX 6

On-column extraction-cleanup method for animal fat (Cahill *et al.*, 1970) used for the extraction of *o,p'*-DDT residues from Japanese quail subcutaneous fat and uropygial gland tissues

1. Up to 0.25 g of frozen fat or uropygial gland tissue was placed on preweighed aluminum foil and weighed to the nearest 0.1 mg.
2. The tissue sample and foil were transferred to a 10 cm activated Florisil column which had been capped with 1.25 cm of Na_2SO_4 and prewetted with 50 ml of hexane.
3. The column walls were rinsed with 25 ml of hexane and the fat of the sample allowed to dissolve in this rinse for 5 min. The 25 ml was eluted at 1 drop/3 sec.
4. When the previous rinse had entered the column bed the column walls were rinsed 3 times with 5 ml volumes of hexane.
5. The column was eluted with 200 ml of 5% diethyl ether in hexane at 1 drop/sec.
6. Steps 6 and 7 of Appendix 4 were carried out.
7. The foil and connective tissues were recovered from the top of the column and reweighed to calculate the amount of fat that had entered the column bed.